Involvement of the Mitotic Kinase Aurora A in DNA Damage Response

Dissertation
Zur
Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. Nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Agnieszka Krystyniak

aus
Polen

Promotionskomitee

Prof. Dr. Josef Jiricny
Dr. Stefano Ferrari (Leitung der Dissertation)
Prof. Dr. Michael Hengartner

Zürich, 2006

TABLE OF CONTENTS

ACKNOWLEDGMENTS

First I would like to thank Stefano Ferrari, my boss and supervisor, for having me as his student. For dealing with my problems and moods, for being happy for my nice results and not upset for the ones which did not want to work at all. For being the person I could talk to, talk not only about the work…

To Joe Jiricny for his support, always having a good word for me and for believing in me, despite sometimes I probably didn't deserve it.

To Bozena Kaminska, my first boss, the person who teached me how to hold a pipette, the person who showed me all the faces of science, the person I could always ask for help and always get it, finally the person who proved and is proving day by day that some things ARE possible.

The biggest thank you goes to the other members of Stefano's lab: Betta, Mahmoud, Christiane, Kim, Ines, Berina. Thank you for your help, when I needed it, thank you for your support when I wanted to through my gels away, thank you for the atmosphere you created.

To the people specially close to me
Patrick – for your help always and without asking for, for "a little bit of magic", different perspective to life and ginger tee ☺
Silvi – for bringing me back to reality, this way or another
Chris – you made me able to fight one of my biggest fears – I will never forget that
Ludo, Ines – for being there for me
Dennis – for making me laugh when I needed it the most, for being serious when I didn't want to laugh anymore…
Nina – Nutella pancakes will always make me think of you ☺
Massimo, Kai, Babsi, Betta – you make each and every lunch break unforgettable ☺
Lidija – for Rotterdam and endless hours on Skype
Christiane – for being much more than a work colleague, for hours of "exchanging information", for always-helpful hands
Thanks to other members of the IMCR: Giancarlo, Anne, Elda, Petr, Franziska, Katja, Tracy, Raj, Pavel, Sybille, Javier, Daniela, Jawad, Tobi, Emilija, Milica, Ippa, Peter, Elisa, Tobias, Christine, Mariane, Jana, Jacob, Reto, Patrick Greiner, Orlando, Malika, Farah, Najad, and all the former members of the institute, without you guys my time here would be just empty…

To Agnieszka – bez Ciebie, gluptasie, wszystko byloby inne. I obawiam sie, ze zwariowalabym calkiem z dala od domu w obcym kraju… Dzieki za kazda kawke, za kazdy telefon, za kazda, mniej lub bardziej powazna rozmowe – nawet nie wiesz, ile to dla mnie znaczylo.

To all those, who were always close to me, even being far, far away…

Prace dedykuje Mamie

SUMMARY

The major mission of the cell division is a faithful and complete duplication of the genome with equal partition of chromosomes into subsequent cell generations. Progression through different stages of the cell cycle is governed by the activity of several members of the Cyclin-dependent kinase family, each pairing with the separate class of Cyclin. In higher eukaryotes, transition from G2 to M phase and completion of mitosis requires action of Cyclin B paired with Cdk1. Cdk1 is tightly regulated by phosphorylation/dephosphorylation processes and only full activation of Cyclin B/Cdk1 complex triggers the initiation of mitosis. Although the role of Cdk1 is crucial and Cdk1 remains the major regulator of mitosis, recent studies have broadened our knowledge of cell division, revealing the presence and importance of other protein kinases. Centrosome maturation and chromosome segregation requires the action of Polo-like kinases, whereas separation of centrosomes seems to be regulated by the kinase Nek2. An important role in centrosome separation, chromosome bi-orientation and cytokinesis has been postulated for Aurora family members.

Genomic stability is under constant threat not only from the products of normal cellular metabolism, but also from radiation and chemicals present in the environment. To ensure proper cell division upon the occurrence damage to DNA, checkpoints are triggered and result in slowing or stopping cell cycle transitions. This, in turn, enables the repair of damage or, when it is too extensive, facilitate the triggering of cell death. Normal cells posses a full complement of cell cycle checkpoints, whereas cancer cells, in most cases, acquire mutations that result in bypass of the checkpoints. Nonetheless, the G2 checkpoint is operative also in cancer cells, since division with incompletely duplicated or damaged DNA is incompatible with life. At the molecular level the G2 checkpoint prevents cells from entering mitosis through inactivation of Cyclin B/Cdk1. This occurs in an ATM/ATR-dependent manner and involves Chk1/Chk2-mediated sub-cellular sequestration and inhibition or degradation of members of the Cdc25 family of phosphatases, which normally activate Cdk1 at the G2/M boundary. It has been recently discovered that kinases involved in spindle formation, like Plk1, Nek2 and Aurora B, play important roles in the cellular response to DNA damage.

In the studies presented in this dissertation I used etoposide, a topoisomerase II poison, to create double strand breaks in DNA, in order to elucidate the role of Aurora A in the DNA damage response. I found that Aurora A indeed is one of the targets of the double-strand break response in G2/M phase (Krystyniak A. et al, 2006). The kinase activity of the enzyme was actively inhibited upon etoposide treatment and this was accompanied by prolonged accumulation of the protein. I also addressed the issue of dependence between Aurora A and Cdk1, showing that the former is not downstream of the latter, but rather inhibition of Aurora A and Cdk1 by DNA damage occurs independently. By using caffeine, an ATM/ATR inhibitor, I showed that Aurora A de-activation in response to DNA damage was dependent on those kinases. More precisely, using cell lines deficient in ATM or conditionally expressing kinase-dead ATR, I was able to confirm that signalling to Aurora A was mediated through ATM. Further, by means of specifically blocking Chk1 with its inhibitor UCN01 or by using siRNA to Chk1, I showed that the signals were delivered to Aurora A via a Chk1-dependent pathway. Those results were additionally confirmed by experiments with cells functionally deficient in Chk2 (HCT15). Looking for the mechanism responsible for Aurora A inhibition, I found that the point mutation S342 to A resulted in a mutant that was active and could not be inhibited by DNA damage. S342 was already postulated as a negative site and it is located directly next to one of the binding motifs for PP1 – a known Aurora A interaction partner. Upon etoposide treatment, however, the interaction between Aurora A and PP1 is highly diminished (Krystyniak A. et al, manuscript submitted). Using the point mutant A342 of Aurora A I found that mutation to non-phosphorylable alanine prevents releasing of the phosphatase from the complex. On the contrary, mutation of the same site to aspartic acid, to mimick constitutive phosphorylation, resulted in complete abolishment of binding, irrespective of the presence of DNA damage.

Finally, I took advantage of two independent approaches to examine the possibility that reconstitution of Aurora A activity in DNA damaged cells may trigger mitotic cell division. Transient transfection of cells with active (wild-type or S342A) but not with inactive (kinase-dead or S342D) forms of Aurora A, enabled them to bypass the DNA damage-induced cell cycle arrest and proceed to mitosis. To avoid large overexpression of proteins in transfection assays and given that such method requires time for the protein to be expressed, I used a more appropriate approach. This allows rapid transduction of proteins in the cell in a manner compatible with the kinetic of the G2 arrest in response to DNA damage. To this end, I directly transduced active AuroraA isoforms into G2-arrested

cells, immediately after inducing double-strand breaks in DNA. This resulted in mitotic entry, despite the unrepaired damage. A closer look to the molecular mechanism underlying progression to mitosis in these conditions revealed that active Aurora A promoted reactivation of Cdk1, thus indicating that Aurora A plays a key role upstream of Cdk1, at least under DNA damage conditions.

Aurora A, similarly to other members of the family, is known to be regulated by phosphorylation. Phosphorylation of a conserved residue, T288, localized in the activation loop of the catalytic domain of the kinase, results in significant increase of Aurora A kinase activity. I was able to show that phosphorylation of T288 may occur through an intermolecular autophosphorylation mechanism. Moreover, I found that PKA, previously claimed to be the kinase responsible for this event *in vitro*, was not involved in T288 phosphorylation *in vivo.* Surprisingly phosphorylation of this site, which is a direct indicator of the activity of the kinase, was found also in DNA-damaged cells to an extent comparable, or even higher than in mitotic cells. This situation is reminding of Cdk1, where phosphorylation at the T-loop residue T161 is hierarchically less important than inhibitory phosphorylation at the ATP-binding site. This finding points to presence of other, maybe structural, mechanisms responsible for the activity of Aurora A that remain to be discovered.

ZUSAMMENFASSUNG

Die Hauptaufgabe der Zellteilung ist die korrekte und komplette Verdoppelung des Genoms und die folgende gleichmässige Aufteilung der Chromosomen auf die beiden Tochterzellen. Dieser Prozess wird durch eine Familie von Cyclin-abhängigen Kinasen dirigiert. Jede Kinase verbindet sich mit einer eigenen Klasse von Cyclinen, welche benötigt werden, um die verschiedenen Stadien des Zellzyklus zu durchlaufen. In höheren Eukaryoten sind der Übergang von der G2- zur M-Phase und der Abschluss der Mitose abhängig von der Aktivität eines Komplexes aus Cylin B und Cdk1. Die Aktivität von Cdk1 ist sehr straff reguliert durch Phoshorylierung und Dephosphorylierung. Die volle Aktivierung von Cyclin B/Cdk1 leitet die Mitose ein. Aktuelle Studien haben das Wissen über die Zellteilung erweitert und aufgezeigt, dass neben Cdk1 auch weitere Proteinkinasen wichtige Funktionen haben. Die Centrosomen-Reifung und die Aufteilung der Chromosomen erfordert die Beteiligung von Polo-ähnlichen Kinasen. Die Aufteilung der Chromosomen scheint dahingegen von Nek2 reguliert zu sein. Eine weitere wichtige Rolle in der Aufteilung der Chromosomen, der Orientierung der Chromosomen und der Cytokinese ist für Mitglieder der Aurora Familie postuliert worden.

Die genomische Stabilität wird nicht nur durch den normalen Metabolismus, sondern auch durch verschiedenste Chemikalien und Strahlung ständig gefährdet. Um die korrekte Zellteilung auch in solchen Fällen zu gewährleisten, bremsen Zellzyklus Kontrollpunkte den Zellzyklus ab oder stoppen ihn ganz. Dadurch wird eine Reparatur des Schadens möglich. Falls das Ausmass des Schadens zu gross ist, wird die Apoptose eingeleitet. Der G2-Kontrollpunkt verhindert, dass Zellen die Mitose beginnen, falls DNS Schäden vorhanden sind. Diese Schäden können während der G2-, oder früher in der S- oder sogar in der G1-Phase entstanden sein, falls die Schäden dann nicht repariert worden sind. Phosphatasen der Cdc25-Familie, welche normalerweise Cdk1 an der G2/M-Grenze aktivieren, werden in solchen Fällen inhibiert und degradiert. Ausserdem vermitteln ATM/ATR und Chk1/Chk2 ein Signal, welches zu einer Sequestrierung des Cyclin B/Cdk1 Komplexes in verschiedenen subzellulären Kompartimente führt. Kürzlich wurde gezeigt, dass Kinasen, wie Plk1, Nek2 und Aurora B, welche in die normale Spindel

Formation involviert sind, auch eine wichtige Rolle in der Zellantwort auf DNA Schäden spielen.

In dieser Dissertation wurde Etoposid, ein Topoisomerase II Inhibitor, benutzt, um Doppelstrangbrüche zu erzeugen, um die Rolle von Aurora A in der DNS-Schadensantwort zu untersuchen. Wir haben herausgefunden, dass die Aktivität von Aurora A durch die Zellantwort auf Doppelstrangbrüche in der G2/M-Phase reguliert wird (Krystyniak A. et al, 2006). Nach Behandlung mit Etoposid war die Kinaseaktivität des Enzyms inhibiert und sein Proteinlevel stieg an. Die Frage war, ob Aurora A abhängig ist von Cdk1 oder umgekehrt. Wir haben herausgefunden, dass die Inhibition von Aurora A und Cdk1 unabhängig voneinander auftritt. Dass die Deaktivierung von Aurora A durch die ATM/ATR Kinasen bewerkstelligt wird, konnten wir durch den ATM/ATR Inhibitor Koffein zeigen. Ausserdem konnten wir durch Blockierung von Chk1 mit dessen Inhibitor UCN01 und siRNA zeigen, dass das Signal an Aurora A über einen Chk1-abhängigen Signalweg geleitet wird. Diese Resultate wurden zusätzlich durch Experimente mit einer Zelllinie, welche defizient ist in Chk2 (HCT15), verifiziert. Eine Punktmutation in Aurora A, S342 zu A, führt zu einem aktiven Enzym, das nicht mehr durch DNS-Schäden deaktiviert werden kann. Die S342 Phosphorylierungsstelle wurde bereits früher als eine negative Regulationsstelle postuliert. Diese Stelle liegt direkt neben einem der Bindemotive für PP1, einem Aurora A Interaktionspartner. Nach Behandlung mit Etoposid verringert sich die Interaktion zwischen diesen zwei Proteinen sehr stark (Krystyniak A. et al, manuscript submitted). Die S342 Mutation zu Alanin, das nicht phosphoryliert werden kann, verhindert die Ablösung der Phosphatase vom Komplex. Auf der anderen Seite haben wir herausgefunden, dass eine Mutation an derselben Stelle zu einem Aspartat, welches eine ständige Phosphorylierung imitiert, die Bildung des Komplexes aus PP1 und Aurora A verhindert.

Zwei verschiedene Ansätze wurden gewählt um die Frage zu untersuchen, ob Aurora A in vorhergehend geschädigten Zellen eine mitotische Zellteilung einleiten kann. Transiente Transfektion von Zellen mit aktiven Formen von Aurora A (Wildtyp oder S342A, aber nicht mit dem kinase-inaktiven S342D Aurora A) hat den Zellen nicht erlaubt die Zellzyklusblockade, welche durch DNS Schäden induziert wurde, zu überwinden und mit der Mitose fortzufahren. Da eine Transfektion auch experimentelle Nachteile mit sich bringt, wie lange Transkriptions- und Translationszeiten oder eine Überexpression des Proteins, wurden direkt aktives Protein in Zellen transduziert, welche in der G2 Phase arretiert waren, gleich nachdem Doppelstrangbrüche induziert wurden. Dies hat den Zellen

ermöglicht sogleich mit der Mitose zu beginnen, obwohl die Schäden noch nicht repariert worden waren. Molekular betrachtet ergibt sich, dass aktives Aurora A die Wiederaufnahme des Zellzyklus über eine Reaktivierung von Cdk1 erwirkt. Daher spielt Aurora A eine Schlüsselrolle im Signalweg oberhalb von Cdk1 - mindestens solange DNS Schäden bestehen.

Aurora A wird ebenso wie andere Mitglieder dieser Proteinfamilie durch Phosphorylierung reguliert. Die Phosphorylierung einer konservierten Aminosäure von Aurora A, Threonin 288, ergibt eine beträchtliche Steigerung der Kinaseaktivität. Dieser Baustein befindet sich in einer Schlaufe in der katalytischen Domäne. Wir konnten zeigen, dass die Phosphorylierung von T288 möglicherweise durch einen intermolekularen Autophosphorylierungsmechanismus stattfindet. Ausserdem konnten wir zeigen, dass PKA *in vivo* nicht involviert ist in die Phosphorylierung von T288, obwohl diese Kinase früher *in vitro* als dafür verantwortlich befunden wurde. Wie niemand gedacht hätte, wurde eine Phosphorylierung dieser Stelle auch in DNS geschädigten Zellen gefunden. Das Ausmass der Phosphorylierung war dasselbe wie in mitotischen Zellen. Da Aurora A unter solchen Umständen nicht aktiv ist, wie wir durch unsere Kinase Tests zeigen konnten, müssen auch andere Mechanismen in der Steuerung der Aktivität eine Rolle spielen.

“The world is full of obvious things which nobody by any chance ever observes”

Arthur Conan Doyle

INTRODUCTION

1. PROTEIN KINASES AND THE MECHANISM OF PHOSPHORYLATION

Protein phosphorylation is the most widespread and well-studied signaling mechanism in eukaryotic cells. Phosphorylation can regulate almost every property of a protein and is involved in all fundamental cellular processes.
Phosphorylation is controlled by protein kinases, which transfer a phosphate group from a donor, ATP or GTP, onto acceptor amino acid in the substrate protein, and protein phosphatases that catalyze hydrolysis of the phosphoester bond and release of the free phosphate (Ferrari S., 2006).

Ever since the discovery of the fact that reversible phosphorylation regulates the activity of glycogen phosphorylase more than 50 years ago, there has been a systematic increase of interest in the role of protein phosphorylation in the regulation of protein function. The near-completion of a human genome allowed the identification of almost all human protein kinases. The most recent kinase phylogenic tree, so-called human kinome consists of 518 kinases, which accounts for 1,7% of all human genes (Manning G. et al, 2002). The current human kinome map is shown in Figure 1.

Most protein kinases belong to a single superfamily named eukaryotic protein kinase (ePK) and share a common catalytic domain. However 13 atypical protein kinase (aPK) families, encompassing 40 genes, have also been identified. These contain proteins reported to have biochemical kinase activity, but which lack sequence similarity to the ePK domain, and their close homologs (Manning G. et al, 2002).
In total 40 aPK in 13 families and 478 ePK, subdivided into 9 major groups, 134 families and 189 sub-families, have been identified (see Figure 1.).

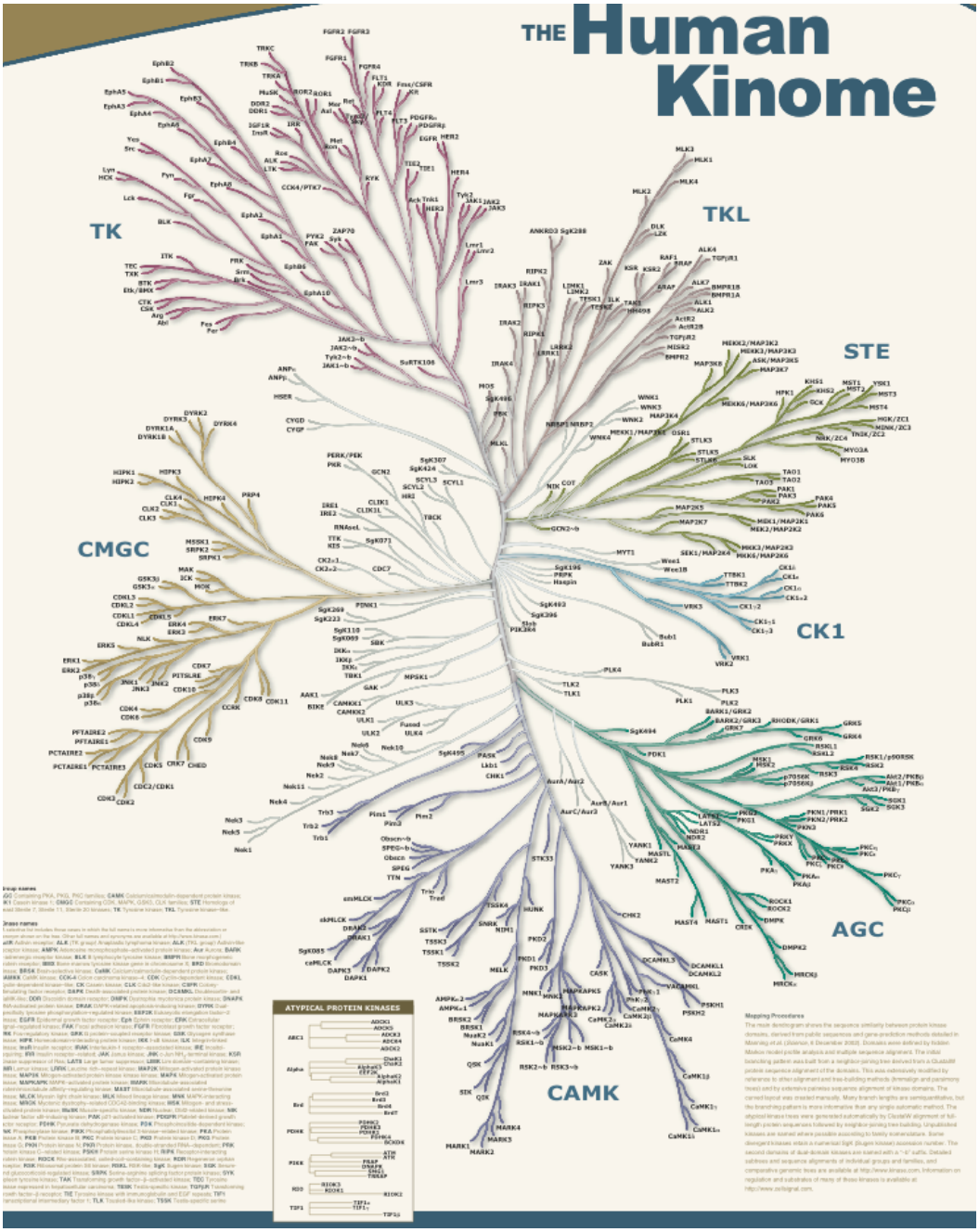


Figure 1. **Human kinome**. Most protein kinases belong to a single superfamily of enzymes whose catalytic domains are related in sequence and structure. The main diagram illustrates the similarity between the protein sequences of these catalytic domains. Each kinase is at the tip of a branch, and the similarity between various kinases is inversely related to the distance between their positions on the tree diagram. Most kinases fall into small families of highly related sequences, and most families are part of larger groups. The seven major groups are labeled and colored distinctly. Other kinases are shown in the center of a tree, colored grey. The inset diagram shows trees for seven atypical protein kinase families. These proteins have verified or strongly predicted kinase activity, but have little or no sequence similarity to members of the protein kinase superfamily (after Manning G. et. All, 2002, property of Cell Signaling Technology)

From a structural point of view the ePK catalytic domain comprises 12 sub-domains containing highly conserved amino acid residues. All ePKs display a similar fold: they consist of a N-terminal lobe, predominantly composed of β-sheets and one single α-helix called the C-helix, and a larger C-terminal region that is essentially made up of α-helices (Ferrari S., 2006). The amino-terminal lobe contains subdomains I to IV, whereas rest of them (subdomains VI to IX) belong to the carboxy-terminal lobe. In between the two lobes there is a deep cleft which accommodates ATP (Johnson L.N. and Lewis R.J., 2001). Schematic structure of a catalytic domain of ePKs is shown in Figure 2.

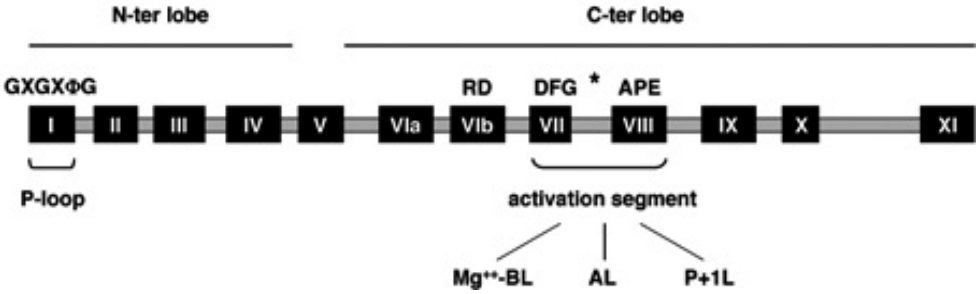


Figure 2. **Structure of protein kinases' catalytic domain.** The core catalytic domain of protein kinases encompasses 12 subdomains I-IV and VI-IX that participate in the formation of the bi-lobate structure of the protein kinase. Conserved residues in the P-loop and in the activation segment are shown. The asterisk represents the position of residue(s) in the activation segment the prosphorylation of which triggers kinase activation (after Ferrari S., 2006)

Bound ATP is capped by a glycine-rich motif called phosphate loop or P-loop, located in subdomain I. P-loop displays the conserved motif GXGXΦG, where Φ is in most cases a hydrophobic amino acid. Access to the ATP-binding site in many kinases is regulated by a peptide located between the conserved motifs DFG and APE in subdomains VII and VIII. This region, called the activation segment, being about 20-30 residues in length, contains one or two phosphorylation sites critical for activation of the kinase. In most cases phosphorylation of the activation segment causes conformational change of the loop, resulting with its interaction with the substrate (Huse M. and Kuriyan J., 2002). Structurally, the activation segment contains three elements, the Mg^{2+}-binding loop, the

activation loop and the P + 1 loop. The first is involved in chelating the Mg^{2+}-ATP complex, the second physically contains the site(s) of regulatory phosphorylation, whereas the third plays a role in substrate binding (Nolen B. et al, 2004).

Protein kinases, being among the largest families of genes in eukaryotes, mediate most of the signal transduction in eukaryotic cells. By modifications of substrate activity, protein kinases also control many other cellular processes, including metabolism, transcription, cytoskeletal rearrangements and cell movement, apoptosis, differentiation or finally cell cycle.

2. CELL CYCLE

2.1. Cyclin-dependent kinases

The Cyclin-dependent kinases (Cdks) are heterodimeric complexes composed of a catalytic kinase subunit and a regulatory subunit. They compromise a family divided into two groups based on their roles in the cell cycle progression and transcriptional regulation.
Members of the first group are the core components of the cell cycle machinery and include Cyclin D-dependent kinase 4 and 6, Cyclin E-dependent kinase 2, Cyclin A-dependent kinase 1 and 2 and Cyclin B-dependent kinase 1. Together, these Cyclin/Cdk complexes are the universal cell cycle regulators, with each complex controlling a specific transition between the subsequent phases in the cell cycle (Smits V.A.J. and Medema R.H., 2001). As anticipated above activation of the Cdk requires binding to a specific regulatory subunit, termed a cyclin. Originally, cyclins where named after their fluctuating levels through the cell cycle. Cyclin binding is, however, not sufficient to trigger Cdk activation. Cyclin-dependent kinases are regulated by positive phosphorylation by Cdk-activating kinase (CAK) as well as negative phosphorylation events, but also by their association with endogenous Cip/Kip or other inhibitors (eg. INK4 – inhibitor of Cdk4).

In contrast to the first group of Cdks, the second group, including Cyclin H-Cdk7 and Cyclin T-Cdk9 (pTEFb), promote initiation and elongation of nascent RNA transcripts by

phosphorylating the carboxy-terminal domain (CTD) of RNA polymerase II (Shapiro G.I., 2006). Schematic explanation of the main role of Cyclin H-Cdk7 and Cyclin T-Cdk9 is shown in Figure 3.

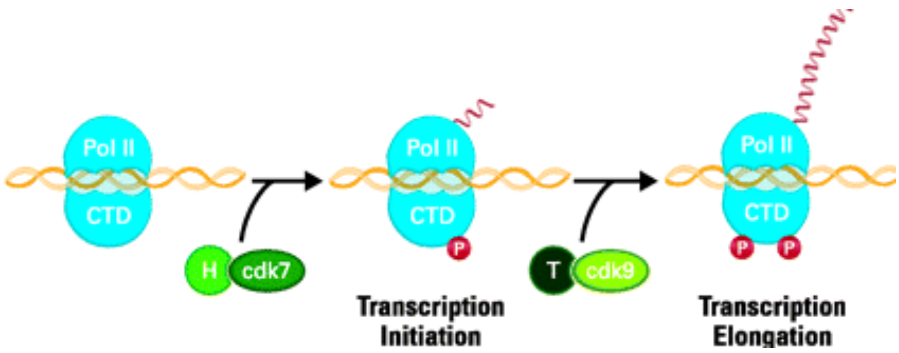


Figure 3. **Main action of Cdk7 and Cdk 9.** The transcriptional Cdks 7 and 9 phosphorylate the carboxy-terminal domain (CTD) of RNA polymerase II (Pol II), facilitating the initiation of transcription and efficient elongation (after G. I. Shapiro, 2006)

Inhibition of these Cyclin-dependent kinases preferentially affect mRNAs with short half-lives, including those encoding anti-apoptotic proteins, cell cycle regulators, and p53, and NFκB (nuclear factor kappa B) pathway components, as well as VEGF (vascular endothelial growth factor).

Cdk9, which associates with one of the Cyclins T or Cyclin K to form a complex called positive transcription elongation factor b (P-TEFb), has also been reported to phosphorylate tumor suppressor protein p53, specifically Ser33 on the N-terminus and, Ser315 and Ser392 on the C-terminus. However, the precise biological role of this phosphorylation remains unclear (Radhakrishnan S. K. and Gartel A. L., 2006)

2.1.1. Cyclin-dependent kinases in the cell cycle control

The major mission of the cell division is a faithful and complete duplication of the genome followed by an equal partitioning of chromosomes to subsequent cell generations (Lukas J. et al 2004).

The replication cycle of a typical eukaryotic, somatic cell consists of four phases: G1, S (DNA synthesis), G2 and M (mitosis). The result of this process is the generation of two daughter cells that are equivalent both in genetic "inside" and in size to the parental cell.
Early studies with cultured mammalian cells concluded, that progression through the cell cycle was governed by several families of cyclin-dependent kinases, each pairing with a separate class of cyclin (Nigg E. A., 2001). The cell cycle progression requires a different cyclin-dependent kinase for progression through each stage of the cell cycle, as shown in the Figure 4.

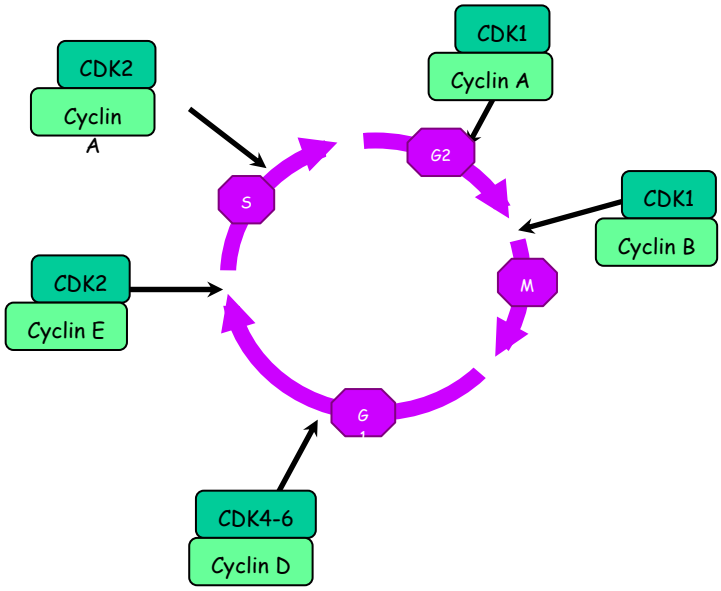


Figure 4. **Cell cycle progression with responsible cyclin-dependent kinases** (explanation in the text)

Extracellular stimuli up-regulate Cyclin D that, upon binding to Cdks 4 and 6, which stimulates quiescent cells to enter the cell cycle, whereas Cyclin E/Cdk2 complexes regulate the G1/S transition. Cyclin A is induced shortly after Cyclin E and binds Cdk2 in S-phase and Cdk1 in G2 and mitosis. Cyclin A is involved in the regulation of S-phase entry and later on is found to be important in G2 and M phase. The entry into mitosis is under the control of B-type cyclins, which also associate with Cdk1 (Smits V.A.J. and Medema R.H., 2001)
I will describe the particular stages of the cell cycle and the detailed roles of different Cdks in more details in following chapters.

2.1.1.1. G1 progression and G1-to-S transition

In response to mitotic stimulation, cells synthesize D-type cyclins that assemble with Cdks 4 and 6 in process that requires contribution of a Cip/Kip family member. Studies using cultured cells have indicated that passage through the G1/S transition is regulated by Cyclin D/Cdk4/6 and their phosphorylation of retinoblastoma susceptibility protein – Rb. In its hypophosphorylated state, Rb prevents progression from G1 to S through its interaction with E2F transcription family members. This interaction not only blocks transcriptional activation of E2F, but also actively represses transcription through the recruitment of histone deacetylases to the promoters of genes required for S-phase entry (Harbour J. W. and Dean D. C., 2000). The phosphorylation of Rb in G1 by Cyclin D/Cdk4/6 (and subsequently by Cdk2) is a requisite event in reversing the repressive effects of Rb and de-repressing transcription of a number of genes required for exit form G1 and initiation and completion of S-phase. It has been further suggested that phosphorylation of Rb by Cyclin D/Cdk4/6 initiates a subsequent round of phosphorylation of the tumor suppressor by Cdk2, which suggests lack of overlapping functions between ckd4/6 and Cdk2. Therefore Cyclin D-dependent kinase facilitate G1 progression by first phosphorylating Rb, relieving transcriptional repression by the Rb-E2F complex, and by sequestering Cip/Kip proteins, facilitating activation of Cyclin E/Cdk2.
G1 progression is negatively regulated by members of the INK4 family, which are known to be specific inhibitors of Cdks 4 and 6. p16 ^{INK4A} accumulates as cells age and induces G1 arrest during senescence by associating with Cdks 4 and 6 and promoting release of D-type Cyclins. The subsequent destabilization of D-type cyclins and the redistribution of Cip/Kip proteins to Cdk2 contribute to trigger the permanent G1 arrest that precedes senescence (Sherr. C.J. and Roberts J. M., 1999).

Cyclin E/Cdk2 mediated Rb phosphorylation disrupts the binding of Rb to E2F, allowing E2F activation and therefore the transcription of genes necessary for S-phase entry and progression, including Cyclin E itself (Geng Y. et al, 1996). Besides Rb, Cyclin E/Cdk2 phosphorylates also other targets, including $p27^{Kip1}$, the degradation of which further facilitates S-phase entry (Sheaff R. J. et al, 1997), p220 nuclear protein mapped to ATM (Ataxia Teleangiectasia) locus (^{NPAT}) (Zhao J. et al, 1998), which stimulates replication-dependent histone gene transcription, and nucleophosmin, which regulates centrosome duplication (Okuda M. et al, 2000).

2.1.1.2. S-phase progression

Our current understanding of the mechanism controlling S-phase progression is centered on evidence for a key role played by the Rb pathway. As mentioned above, after Cdk-mediated phosphorylation of Rb during G1-phase, E2F activity is being freed and E2F protein is released. E2F binds to its heterodimeric partner - DP-1 - and thereby directs transcription of genes required for the S-phase. This transcription, however, is activated only transiently. Further S-phase progression requires the down-regulation of E2F-1 activity, which is, in part, due to the Cdk-mediated phosphorylation (Kitagawa M. et al, 1995).

Several Cdk holoenzymes phosphorylate E2F-1 during the S and G2 phases and participate in the appropriately timed neutralization of its activity (Reis T. and Edgar B. A., 2004). Cyclin A/Cdk2 stably interacts with the N-terminus of E2F-1 and directs the phosphorylation of both E2F-1 and DP-1. Phosphorylation of both components inhibits the binding activity of the dimer and releases it from the DNA. On the other hand phosphorylation of E2F-1 by Cyclin A/Cdk1 at Ser375 promotes the formation of the complex of E2F-1 with Rb, thus keeping the E2F-1 inactive late in the cell cycle (Peeper D.S. et al, 1995). Finally, the kinase associated with the RNA polymerase transcription factor II (TFIIH), Cyclin H/Cdk7, phosphorylates E2F-1 at Ser408 and Thr433, which then promotes its degradation via ubiquitination.

Scheme of S-phase progression and the involvement of ckd1, 2 and 7 are shown in Figure 5.

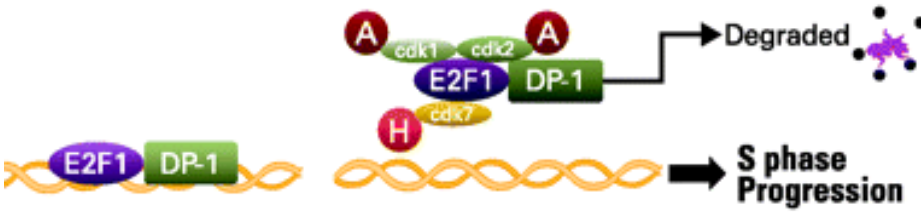


Figure 5. **S-phase progression**. After Rb protein phosphorylation, E2F, along with heterodimeric DP family member partner, directs transcription of S-phase genes. Transcription is activated transiently. E2F-1 activity is in part limited by phosphorylation, mediated by Cyclin A-Cdk2, Cyclin A-Cdk1 and Cyclin H-Cdk7. Appropriately timed neutralization of E2F activity is required for proper S-phase progression (adapted from G. I. Shapiro, 2006)

2.1.1.3. Cdk *inhibitors*

Cdk inhibitors (CKIs) serve as negative regulators of the Cdks (Sherr C.J. et al, 1998). CKIs are classified into two distinct families based on their structural and functional characteristics. The members of the INK4 family of CKIs ($p16^{Ink4a}$, $p15^{Ink4b}$, $p18^{Ink4c}$ and $p19^{Ink4d}$) contain multiple ankyrin repeats and act as negative regulators of Cdk4 and 6 by binding to the catalytic subunit and preventing formation of the active Cyclin/Cdk complex.

The Cip/Kip family of CKIs ($p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$) is more broadly acting and regulates both Cdk4/6 and Cdk2 activity. Each member of the family contains a characteristic motif within the amino-terminal region that enables them to bind to both cyclin and Cdk subunits. The stoichiometry between Cdks and CKIs is important and determines the activity of Rb and the proliferative state of cells.

2.1.1.4. G2/M transition

In higher eukaryotes, completion of the G2/M transition requires two Cyclins, A2 and B1, that pair with Cdk1. The Cyclin B/Cdk1 complex was originally defined as the maturation-promoting factor of M-phase promoting factor (MPF), and was identified in meiotic frog eggs as a factor capable of inducing M phase in immature G2 oocytes. Regulation of Cyclin B/Cdk1 complexes at multiple levels ensures the tight regulation of the timing of the mitotic entry.

After association of the cyclin with its Cdk, the complex is regulated by phosphorylation events, which either activate or inhibit its kinase activity. Phosphorylation of the conserved threonine (Thr161), localized in the T-loop of the enzyme, by Cdk activating kinase (CAK) is required for the activation of Cyclin B/Cdk1 complex. CAK was found to be a Cyclin/Cdk complex itself and its composed of Cyclin H and Cdk7. A third component, MAT1, is thought to stabilize the Cyclin H/Cdk7 interaction (Fisher R.P., 1994).

During G2-phase, mammalian Cyclin B/Cdk1 complexes are held in an inactive state by phosphorylation of Cdk1 at the two negative regulatory sites, threonine 14 (Thr14) and tyrosine 15 (Tyr15). Tyr15 is situated in the ATP-binding site of the Cdk1 and phosphorylation of this residue interferes with the phosphate transfer to a bound substrate

(Atherton-Fessler S. et al, 1993). In contrast, Thr14 phosphorylation inhibits Cdk1 by interfering with its ATP binding (Endicott J.A. et al, 1994). Both sites seem to be crucial for Cdk1 status since replacement of those amino acids with non-phosphorylable ones leads to complete deregulation of Cdk1 and entry into mitosis.
Phosphorylation of Cdk1-Tyr15 is carried out by the Wee1/Mik1 family of protein kinases. A Wee-1 related kinase, Myt1, has been shown to phosphorylate both Thr14 and Tyr15 of Cdk1, but with the strong preference for Thr14 (Liu F. et al 1997). Wee1 was shown to be a nuclear protein, whereas Myt1 is a membrane-associated protein that localizes to endoplasmic reticulum and the Golgi complex (Liu F. et al 1997). The distinct localization of both kinases may guarantee inhibition of differently localized subpopulations of Cdk1.

Cdk1 is, on the other hand, positively regulated by Cdc25 family, acting in opposition to Wee-1 and Myt1. Members of this family were shown to be dual specificity phosphatases, which can dephosphorylate both Thr14 and Tyr15 of Cdk1 (Izumi T. and Maller J.L., 1993). Dephosphorylation of Thr14 and Tyr15 by Cdc25C in late G2 activates the Cyclin B/Cdk1 complex. Cyclin B/Cdk1 in turn phosphorylate Cdc25C, thus forming an autocatalytic feedback loop (Izumi T. and Maller J.L., 1993). Full activation of cyclin B/Cdk1 triggers the initiation of mitosis.

2.1.1.5. M phase

Mitosis is a highly coordinated process in which the two copies of each chromosome (sister chromatids) are segregated away from each other to opposite poles of the cell. Subsequently, the cell is cleaved between the two newly formed nuclei, leading to formation of two independent, daughter cells.

Mitotic division consists of five, tightly regulated phases, namely: prophase, prometaphase, metaphase, anaphase and telophase, followed by cytokinesis.

Figure 6 shows chronology of the events in M-phase and the regulatory factors of each step.

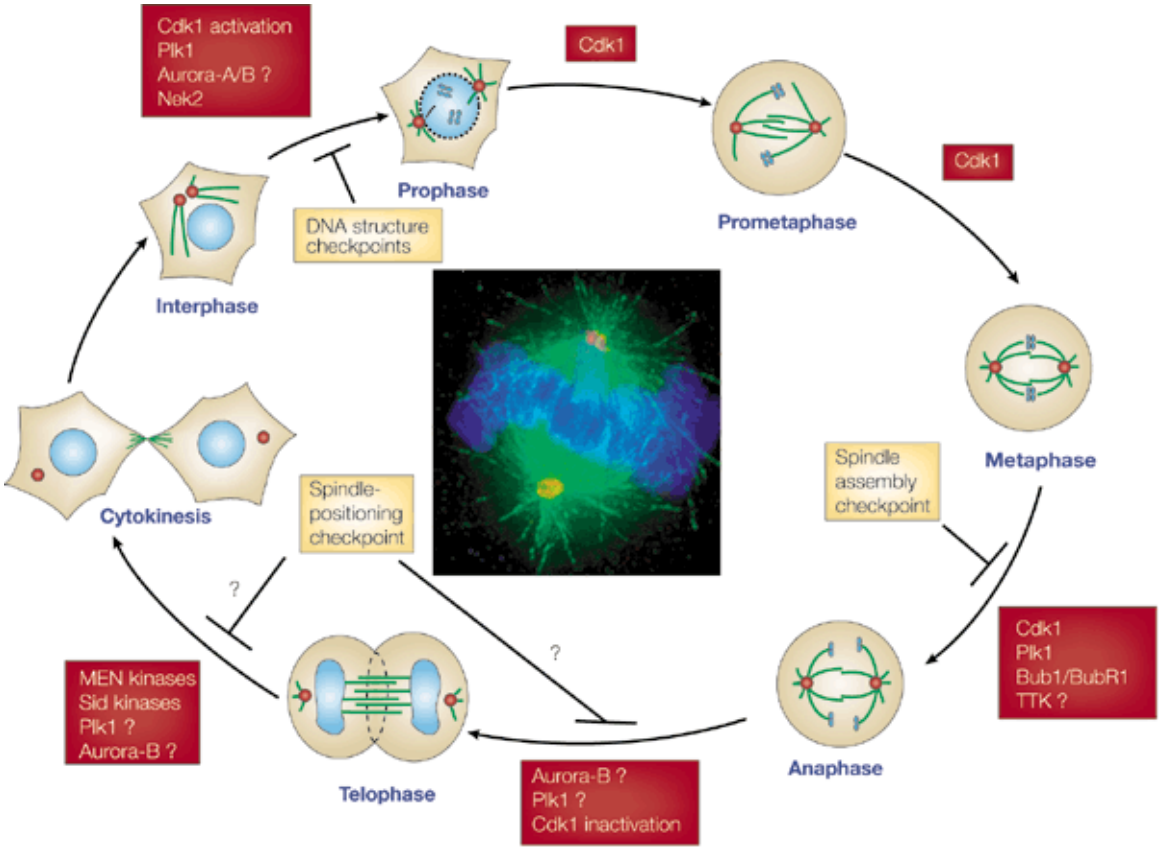


Figure 6. **Chronology of M-phase events** (details in the text) (from Nigg E.A., 2001)

During prophase chromatin condenses into well-defined chromosomes and previously duplicated centrosomes migrate apart forming the poles of the future spindle. Chromatin condensation requires the combined action of a multisubunit protein complex – condensin and of topoisomerase II, the latter being involved in the decatenetion of sister chromatids. In late prophase, the microtubules that formed the interphase cytoskeleton are disassembled, and highly dynamic microtubules radiate from centrosomes (Ferrari S., 2006). These mitotic microtubules drive the migration of separated centrosomes, each with its pair of centrioles, to the opposite poles of the cell. Nuclear envelope breakdown (NEBD) occurs shortly after centrosome separation. During interphase, the nuclear envelope is stabilized by a karyoskeletal structure known as the nuclear lamina, but at the onset of mitosis this structure disassembles as a consequence of lamin hyperphosphorylation (Nigg E. A., 2001). Although lamins can be phosphorylated by many kinases *in vitro*, the predominant kinase triggering mitotic lamina depolimerization *in vivo* is Cyclin B/Cdk1 (Nigg E. A., 1995).

During prometaphase microtubules are captured by kinetochores, specialized structures associated with centromere DNA on mitotic chromosomes. Chromosomes then congress to an equatorial plane, so-called metaphase plate.

Anaphase begins shortly after all chromosomes underwent proper bipolar attachment to the spindle. Its onset is characterized by the simultaneous separation of all sister chromatids as a result of a loss of sister chromatid cohesion. This separation depends on the degradation of an inhibitor called securin, by ubiquitin-dependent proteolysis (Nasmyth K. et al, 2000). Securin prevents a protease, called separase, from abolishing sister chromatid cohesion, by cutting a component of a multiprotein complex known as cohesin. The other important step in cohesin degradation is its phosphorylation, predominantly by Cdk1, but also by Plk1 (Polo-like kinase 1).

Once the chromosomes have arrived at the poles, nuclear envelopes reform around the daughter chromosomes, and chromatin decondensation begins (telophase).

Cytokinesis, the final step of cell division, physically dissociates the two daughter cells from each other (Glotzer M., 2005). Cytokinesis starts with contraction of the equatorial actomyosin ring, leading to membrane furrowing (Scholey J. M. et al, 2003). As furrow ingression completes, the cells remain linked by a cytoplasmic bridge containing the remnant of the spindle midzone, the midbody. The actomyosin ring then disassembles while resolution of the plasma membrane, called abscission, completes cytokinesis (Norden C. et al, 2006).

2.2. Other cell cycle kinases involved in mitotic division

Although Cdk1 remains the major regulator of mitosis, recent studies have broadened our knowledge of cell division, revealing the presence and importance of other protein kinases. A simplified summary is presented in Table 1.

Protein kinase	Function
Cdk1	nuclear membrane breakdown mitotic spindle assembly chromosome condensation APC/C/C regulation
Plk1	centrosome maturation microtubule dynamics chromosome segregation APC/C/C regulation DNA damage recovery
Nek2	centrosome splitting
AurA	centrosome separation Cdk1/Cyclin B1 activation
AurB	chromosome condensation chromosomes bi-orientation cytokinesis
Mps1	centrosome duplication and separation chromatide-spindle attachment
PKA	APC/C/C regulation
PKC	mitotic progression
PI3K	Cyclin B1 expression
Akt/PKB	G2/M checkpoint bypass

Table 1. **Protein kinases controlling the onset and progression through mitosis.** (after Ferrari S., 2006)

Centrosome maturation and chromosome segregation requires the action of Polo-like kinases (Plks), whereas separation of centrosomes seems to be regulated by Nek2 (NIMA-related kinase 2). A role in centrosome separation, chromosome bi-orientation and cytokinesis has been postulated for aurora family members (described in details in Chapter 3).

2.2.1. Polo-like kinase

Polo-like kinases (Plks) play important roles in several stages of cell cycle, including prophase, metaphase and anaphase of mitosis, cytokinesis and G1/S transition as well as in DNA damage response. Flies, budding and fission yeast contain a single Plk family member (Polo, Cdc5 and Plo1, respectively), while humans, mice, frogs and worm have three Plk members, namely Plk1, Plk2 and Plk3 (nomenclature of human proteins).
Plk1 is expressed primarily during late G2 and M phases, where it is involved in the mitotic machinery regulation. Plk2 expression is mostly in early G1, where it is believed to control entry into S-phase, whereas the level of expression of Plk3 seems to be constant during cell cycle progression. Plk3 is thought to play a role in several stress response pathways, including those activated by DNA damage and spindle disruption (Lowery D.M. et al, 2005).

Plk activity is controlled both by the protein abundance and by its intracellular localization. During prophase and metaphase, Plk1 localizes to centrosome and spindle pole bodies, where it is required for spindle assembly and centrosome maturation, probably by phosphorylating some, yet unknown, centrosome associated targets (Goto H. et al 2005), (Sumara I. et al, 2004). It participates in bipolar spindle formation, likely by generating the force that pulls each chromosome towards the spindle pole (Ahonen L.J. et al, 2005)
During late anaphase Plk1 re-localizes to the spindle midzone facilitating microtubule sliding or some aspects of kinetochore dynamics and eventually come to flank the central portion of the cytokinetic bridge (i.e. the midbody) during telophase and cytokinesis. The role of Plk1 postulated at those stages is the activation of the anaphase-promoting complex/cyclosome (APC/C), which triggers exit from mitosis (APC/C and the regulation of cell cycle by protein degradation will be discussed later).

The kinase activity of Plk1 was found to be efficiently inhibited after DNA damage in G2 and mitosis and postulated to be an important target of the DNA damage checkpoint (DNA damage checkpoints will be discussed in next chapters), enabling cell-cycle arrest at multiple points in G2 and mitosis (Smits V.A.J. et al, 2000), although later studies claimed, that Plk1 is not a principal regulator or mediator of the mitotic DNA damage response (Yuan J-H. et al, 2004). There is no doubt, however, that Plk1 controls recovery from a G2 DNA damage-induced arrest by mediating degradation of Wee1 (van Vugt M.A.T.M. et al, 2004 and 2005).
Among Plk1 substrates are: Cdc25C (Toyoshima-Marimoto F. et al, 2002), BRCA2 (Lin H.R. et al, 2003), Myt1 (Nakajima H. et al, 2003), Cyclin B (Toyoshima-Marimoto F. et al, 2001), Wee1 (Sakchaisri K. et al, 2004) and p53, the function of which Plk1 inhibits (Ando K. et al, 2004).

2.2.2. Nek2 kinase

Nek2 (NIMA-related kinase 2) is a mammalian serine/threonine kinase known to posses high sequence homology to NIMA (Never In Mitosis A), a kinase shown to be necessary for mitotic entry in *Aspergillus nidulans* (Fry A.M. et al, 1995). Nek2 activity is regulated by autophosphorylation, but also its physical association with protein phosphatase 1 (PP1) has been reported (Helps N.R., et al, 2000). Nek2 seems to be cell-cycle regulated: the protein level is low in G1 and increases throughout S and G2 (Fry A.M. et al, 1995), then – in prometaphase to metaphase of mitosis - the protein is rapidly degraded in an APC/C/cyclosome dependent manner.
Nek2 localizes to the centrosome where, when overexpressed, induces centrosome splitting by phosphorylating C-Nap1, its interacting partner (Fry A.M. et al, 1998). The possible inhibition of both Nek2 kinase activity and protein level, as contribution to the cell cycle arrest upon DNA damage, has been shown by a single study (Fletcher L. et al, 2004).

2.3. Ubiquitin-mediated protein degradation in the regulation of cell cycle

Ubiquitin-dependent proteolysis by the proteasome plays an essential role in a number of key biological processes, including cell cycle progression, transcription and signal

transduction. In many cases the target protein is first marked for degradation or processing by phosphorylation. The phosphorylated protein is then recognized and ubiqiutinated in a process that requires three proteins. Ubiquitin is first attached to an ubiquitin-activating enzyme (E1) in an ATP-dependent reaction to form a high-energy thiolester bond. The ubiquitin is then transferred from the E1 protein to an E2 ubiquitin-conjugating enzyme, which functions in conjunction with an E3 protein to link ubiquitin to lysine residues in the targeted protein. A specific lysine residue in the conjugated ubiquitin is then attached to a second ubiquitin, and reiteration of this process results in the assembly of a polyubiquitin chain. The polyubiquitinated protein can then be recognized by the 26S proteasome and degraded or processed (Maniatis T., 1999).
E1 and E2 proteins have been identified and characterized, and the later has been shown to comprise a family of related proteins. In contrast, much less is known about E3 enzyme.

Progression through a eukaryotic cell cycle requires tight control of the activity of different factors. Evidence obtained so far has shown that one of the possible ways to control cell cycle progression is to regulate the abundance of key factors, either by controlling their expression or degradation. The list of cell cycle regulatory proteins that are targeted for turnover by ubiquitin-mediated proteolysis is extensive. Some noteworthy examples are listed in Table 2.

Generally, targeted proteins fall into two categories: those for which ubiqiutin-mediated destruction is central to their proper function – for example proteins that temporarily halt cell cycle progression, but need to be destroyed in order for cell cycle to resume, and those whose expression is limited to a specific cell cycle window by proteolysis, but whose destruction does not seem to be intrinsic to function (Reed S.I., 2003).

In terms of the ubiquitylation machinery, the targeting for turnover of cell cycle regulatory proteins seems to be mediated through two distinct alternative strategies: activation of the target itself, or activation of the protein ubiquitin ligase that transfers ubiquitin to a particular class of target. The former strategy allows for selectivity that is dependent on the regulatory context of individual target molecules, whereas the latter allows for concerted and total destruction of populations of target molecules at particular points in the cell cycle (Reed S.I., 2003).

Table 1 | **Cell-cycle targets of ubiquitin-mediated proteolysis**

Substrate	Organism	Ligase	Specificity factor	Cell-cycle function
Securin/Pds1	H.s., S.c., others	APC/C	Cdc20	anaphase inhibitor
Clb2	S.c.	APC/C	Cdc20, Cdh1	cyclin B (mitosis)
Clb5	S.c.	APC/C	Cdc20	cyclin B (S phase)
Cyclin B	metazoan	APC/C	Cdc20, Cdh1	mitosis
Cyclin A	metazoan	APC/C	Cdc20, Cdh1	S phase, mitosis
Cdc20	H.s., S.c.	APC/C	Cdh1	mitosis
Plk/Cdc5	H.s., S.c.	APC/C	Cdh1	mitosis
Aurora A	H.s.	APC/C	Cdh1	mitosis
Dbf4	S.c.	APC/C	Cdc20	S phase
Ase1	S.c.	APC/C	Cdh1	mitotic-spindle dynamics
Nek2A	H.s.	APC/C	Cdh1	centrosome development
Cdc6	H.s.	APC/C	Cdh1	replication
Geminin	metazoan	APC/C	Cdh1	replication licensing
Cin8, Kip1	S.c.	APC/C	Cdh1	mitotic-spindle motor
Xkid	X.l.	APC/C	Cdh1	mitotic-spindle motor
Hsl1	S.c.	APC/C	Cdc20, Cdh1	G2–M transition
Sic1/Rum1	S.c., S.p.	SCF	Cdc4, Pop1/2	G1–S-transition Cdk inhibitor
Far1	S.c	SCF	Cdc4	G1–S-transition Cdk inhibitor
Cdc6/Cdc18	S.c., S.p.	SCF	Cdc4, Pop1/2	DNA replication
Swe1	S.c.	SCF	Met30	mitosis inhibitor
Cln1,2	S.c.	CF	Grr1	G1 cyclins
Gic1,2	S.c.	SCF	Grr1	budding
Cyclin E	H.s., D.m.	SCF	Cdc4, Ago	G1–S cyclin
$p27^{Kip1}$	H.s., M.m.	SCF	Skp2	G1–S-transition Cdk inhibitor
$p21^{Cip1}$	H.s.	SCF	Skp2	G1–S-transition Cdk inhibitor
p130	H.s., M.m.	SCF	Skp2	G1–S-transition inhibitor
Orc1	H.s.	SCF	Skp2	DNA replication
Emi1	M.m.	SCF	β-TrCP	mitosis (APC/C) inhibitor
Wee1	X.l.	SCF	Tome-1	mitosis inhibitor

D.m., *Drosophila melanogaster*; H.s., *Homo sapiens*; M.m., *Mus musculus*; S.c., *Saccharomyces cerevisiae*; S.p., *Schizosaccharomyces pombe*; β-TrCP, β-transducin-repeat-containing protein; X.l., *Xenopus laevis*.

Table 2. **Cell cycle targets for ubiquitin-mediated proteolysis** (after Reed S.I., 2003)

Most commonly, targeted-activated destruction, in the context of the cell cycle, is carried out by a class a protein-ubiquitin ligase known as SCF (for Skp1/Cul1/F-box), whereas alternative forms of a ligase that is known as the anaphase-promoting complex/cyclosome (APC/C) are themselves activated through signaling pathways that are intrinsic to the cell cycle.

Figure 7 shows which of the ligase classes is responsible for the various cell cycle targets of ubiquitylation.

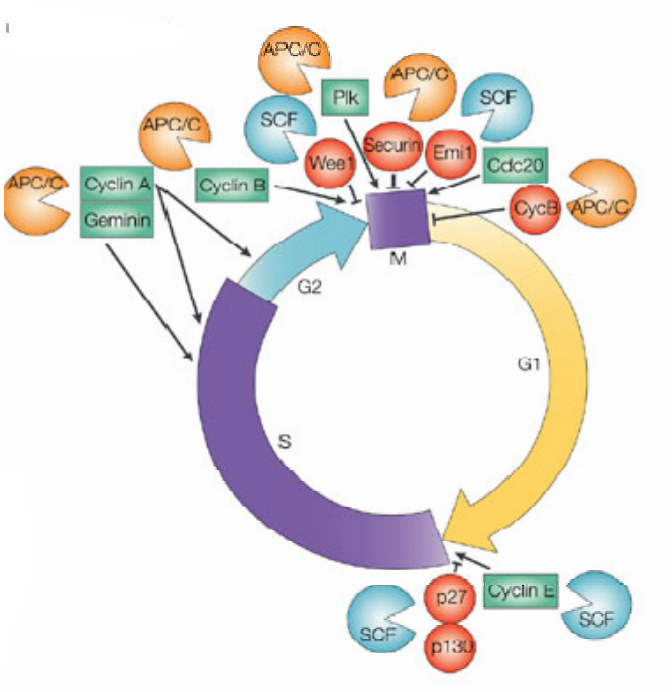


Figure 7. **Key cell-cycle regulatory proteins targeted by ubiquitin-mediated proteolysis.** (after Reed S.J., 2003)

The E3 ubiquitin ligases can be classified into HECT domain, Ring-H2-finger domain and U-box domain superfamilies (Ang L.X., Harper J.W., 2005). Both, SCF and APC/C are members of the Ring-H2-finger-containing E3 ubiquitin-ligase family. SCF complexes are used throughout the cell cycle, whereas APC/C functions primarily during mitosis and G1 phase.

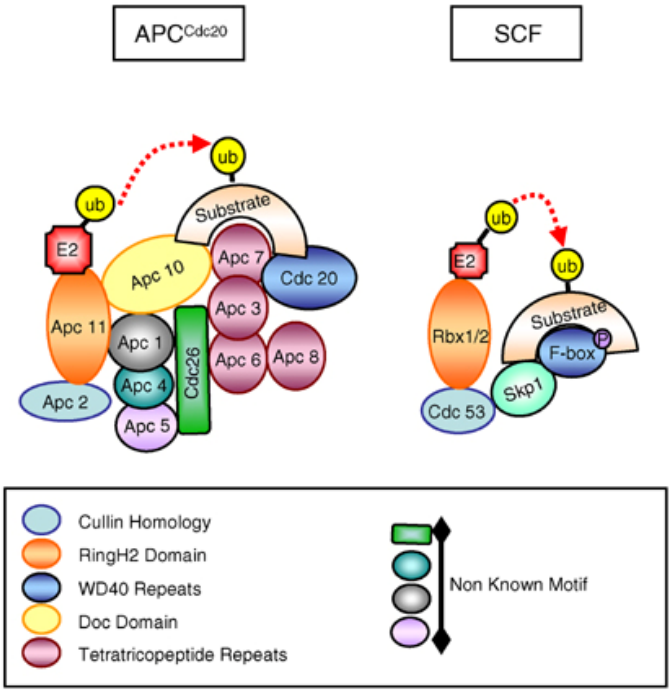


Figure 8. **Comparison of the APC/C^{Cdc20} and the SCF ubiquitin ligases.** Proteins belonging to the same family are identically colored. Interaction of the different APC/C subunits have been represented taking into account the existent results on the association between different APC/C components. (models by Castro A. et al, 2005).

SCF and APC/C differ from each other also in terms of general structure, the APC/C being much larger and composed of different subunits. APC/C and SCF both contain a Cullin, a Ring-H2-finger and a WD40 subunit. The cullin and the Ring-H2-finger proteins form a minimal ubiquitin-ligase module of both E3, and are required for E2 tethering. The APC/C contains additional subunits, such as the tetratricopeptide containing proteins and the Doc protein.
Figure 8 shows schematic comparison of SCF and APC/C composition.

2.3.1. SCF complex

The SCF complex has three primary subunits: Skp1, Cullin and Rbx1/Roc1. This complex can interact with a variety of proteins containing an F-box motif and with Cyclin F, which interacts with Skp1 directly (Bai C. et al, 1996). The Cul1/Rbx1 components form the E3 ubiquitin ligase core that associates with E2 ubiquitin-conjugating enzymes. F-box proteins directly recruit ubiquitination substrates and bridge the interaction between the E2 and the substrate (Feldman R.M. et al, 1997). Thus, the identity of the F-box protein determines the target of the SCF. There are a variety of F-box proteins, including subfamilies with WB40 domains (Fbw) or with leucine-rich repeats (Fbl) (Cenciarelli C. et al, 1999). Moreover, F-box proteins often recognize their substrates in a manner dependent upon particular modification, for example phosphorylation or glycosylation (Feldman R.M. et al, 1997).
Within the context of a complex cellular environment, phosphorylation allows for substrate discrimination by SCF complex due to its ability to specifically recognize phosphorylated motifs called phosphodegrons. A phoshodegron is defined as one or a series of phosphorylated residues on the substrate that directly interact with a protein-protein interaction domain in an E3 ubiquitin ligase (e.g. an F-box protein), thereby linking the substrate to the conjugation machinery (Winston J.T. et al, 1999). Additionally, phosphorylation allows temporal regulation of substrate degradation, even if SCF complexes are constitutively active throughout the cell cycle.
Figure 9 presents examples of protein degraded by SCF in the phosphorylation-dependent fashion.

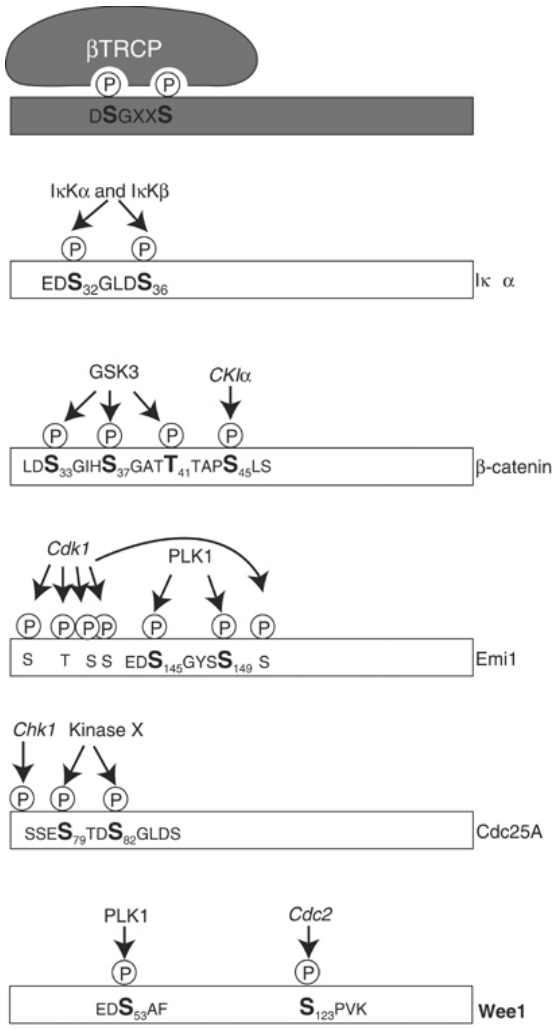


Figure 9. **Multiple kinases regulate the degradation of SCF substrates, including that of β-TRCP.** The priming kinase, depicted in italics, first phosphorylated the substrate to prime it for subsequent binding to the second kinase. The second kinase completes formation of the phoshodegron consensus motif (in bold) by phosphorylation residues that can now be directly recognized by β-TRCP. The absence of the priming activity results in an inability of the second kinase to phosphorylate efficiently the phosphodegron , as well as the stabilization of the substrate. The absence of the second kinase activity also prevents efficient substrate turnover, as the phosphodegron motif remains unphosphorylated and unrecognizable by β-TRCP (after Ang X.L. and Harper J.W., 2005).

The SCF complex itself is also subject to post-translational modifications in the form of neddylation on the Cullin subunit (Lammer D. et al, 1998). Neddylation describes the linkage of Nedd8 – a small 76-residue protein with sequence similarity to ubiquitin – to a lysine residue of the substrate. Neddylation occurs on the winged-helix B domain of Cullin, which is part of the domain that forms a globular tail binding Rbx1 and E2-conjugating enzyme (Zheng N. et al, 2000). Although Nedd8 conjugation is known to dramatically increase ubiquitin transfer by E2s bound to the Rbx1/Cullin complex, the exact mechanism of this stimulation remains unknown.

2.3.2. APC/Cyclosome

The APC/C is a large protein complex containing at least 11 core subunits (Zachariae W. et al, 1998), which can further associate with at least three known different activators. The

majority of those subunits are stably associated throughout the cell cycle, except for the different activators whose binding to APC/C is cell cycle regulated.
Subunits and activators of the APC/C are presented in Table 3.

Core subunit	**Motif**
APC/C1/Tsg24	Rpn1/2 homology
APC/C2	Cullin homology
APC/C3/Cdc27	Tetratricopeptide repeats
APC/C4	
APC/C5	
APC/C6/Cdc16	Tetratricopeptide repeats
APC/C7	Tetratricopeptide repeats
APC/C8/Cdc23	Tetratricopeptide repeats
APC/C10	Doc domain
APC/C11	Ring-H2-finger domain
Cdc26	

Activators	**Motif**
Cdc20/p^{55cdc}	WD40 repeats
Cdh1	WD40 repeats

Table 3. **Subunits of the APC/C in vertebrate cells** (after Castor A. et al, 2005).

The APC/C is fully active only when it is bound to the activator, mainly Cdc20 or Cdh1, resulting in distinct assemblies called APC/C^{Cdc20} or APC/C^{Cdh1}. Since Cdc20 and Cdh1 can bind to APC/C substrates, they may activate ubiquitination reactions by recruiting substrates to the APC/C, but the details of the process are largely unknown.

APC/C-mediated ubiquitination depends on either one or three rather poorly defined sequence elements in the substrate, the destruction box (D-box), the KEN box and recently described GxENbox (Castro A. et al, 2003). The D-box was found to be necessary to induce degradation of Cyclin B (Glotzer M. et al, 1991), Cyclin A (Lorca T. et al, 1992),

Nek2 (Hames R.S. et al, 2001) and Aurora A (Castro A. et al, 2002). The KEN box sequence is present in the APC/C activator Cdc20, which is itself and APC/C^{Cdh1} substrate. A particularly interesting case is degradation of Aurora A. Proteolysis of this protein is exclusively mediated by APC/C^{Cdh1} and requires the presence of a double degradation motif: the D-box and a new degradation signal, named DAD (D-box Activating Domain) or A-box. Mutation of either of these two domains prevents destruction of Aurora A (Castro A. et al, 2002), (Littlepage L.E. and Ruderman J.V., 2002).

Ubiquitin-dependent degradation of the cell cycle factors induced by the APC/C is a key mechanism used by the cell to tightly regulate different transitions throughout cell division. Thus, the APC/C orchestrates mitosis by controlling anaphase entry and progression, exit of mitosis and G1 phase. Moreover it plays an important role in the formation of the prereplicative complexes required to induce DNA replication (Castro A., 2005).

Figure 10 shows the involvement of APC/C in degradation of different cell cycle proteins with activator distinction.

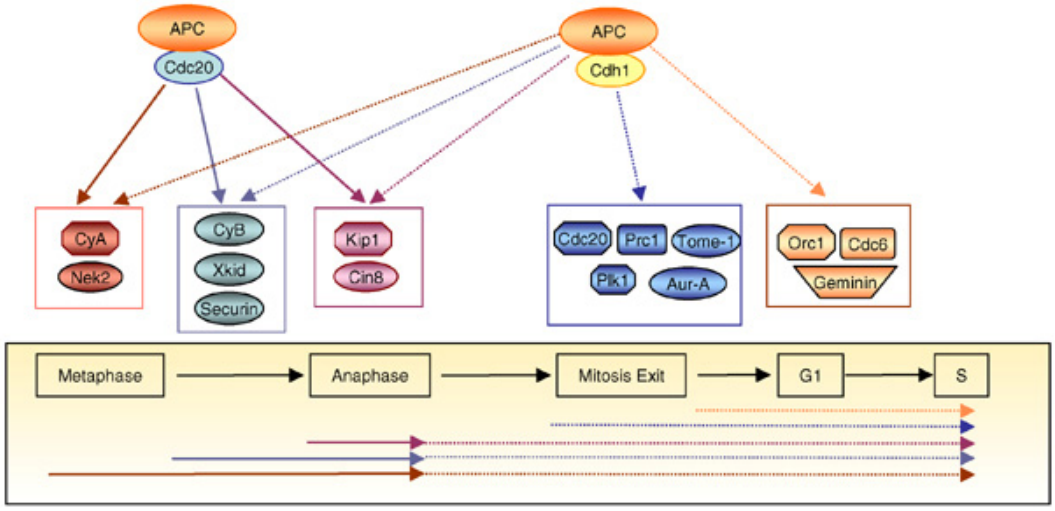


Figure 10. **APC/C^{Cdc20} and APC/C^{Cdh1}-dependent degradation of different cell cycle proteins.** APC/C^{Cdc20} is first activated at the prometaphase-metaphase transition, where it will induce Cyclin A and Nek2 degradation. This complex is also responsible for the subsequent degradation at metaphase of Cyclin B, Xkid and securin and at the anaphase of the kinesin Kip1 and Cin8. From late anaphase until mitotic exit and throughout G1 phase, the degradation of all these proteins is insured by APC/C^{Cdh1}. Moreover, this complex will first induce proteolysis of Cdc20, Prc1, Tome-1, Plk1 and Aurora A at mitotic exit and subsequent degradation of Orc1, Cdc6 and Geminin in early G1 (details in the text) (after Castro A., 2005).

Entry into anaphase is marked by the initiation of sister chromatid separation. The APC/C-dependent degradation of securin enables separase activation and, as a consequence, cleavage of the cohesin complex (Uhlmann F. et al, 2000). Proteolysis of securin is ensured by APC/C^{Cdc20} before anaphase onset, but its degradation is maintained until the end of G1 by APC/C^{Cdh1}.

APC/C also induces degradation of several factors essential for spindle-pole separation and spindle disassembly, for example Xkid. Xkid plays an important role in prometaphase by maintaining the polar ejection force, that pushes chromosomes away from the pole and that mediates chromosome congression (Antonio C. et al, 2000). However, subsequent proteolysis of this protein is also essential to allow chromosome movements to the spindle poles during anaphase. Xkid degradation is mediated both by APC^{Cdc20} and APC^{Cdh1}. Two other motor proteins, the kinesin Kip1 and Cin8, are proteolysed by APC/C. They are both required to separate the spindle poles during spindle assembly and metaphase and their subsequent degradation at anaphase is essential to allow progression through this phase.

Degradation of Cyclin B, the first known APC/C substrate, begins at metaphase and continues throughout mitosis and G1. Both complexes APC/C^{Cdc20} and APC/C^{Cdh1} mediate its degradation. Cyclin B proteolysis is required to inhibit Cdk1 activity and as a consequence to induce different cell processes, such as sister chromatid separation, disassembly of the mitotic spindle, chromosome decondensation, cytokinesis and re-formation of the nuclear envelope (Murray A.W. and Kirschner M.W., 1989), (Luca F.C. et al, 1991), (Gallant P. and Nigg E.A., 1992), (Holloway S.L. et al, 1993), (Surana U. et al, 1993).

Another substrate, whose degradation is carried out by APC/C^{Cdh1}, necessary for mitotic exit is Plk1. It has been shown that destruction of this protein is required to inactivate APC/C-dependent degradation of mitotic cyclins as cells enter S-phase.

The main substrate of APC/C during G1 phase is one of its own activators - Cdc20. Cdc20 proteolysis by APC^{Cdh1} induces APC^{Cdc20} inactivation and allows the switch from APC/C^{Cd20} to APC/C^{Cdh1}. APC/C^{Cdc20} is active in the presence of high Cyclin B/Cdk1 activity, whereas Cdh1 phosphorylation by this complex prevents APC/Cdh1 association, and as a consequence, APC/C^{Cdh1} activation. Once Cyclin B degradation has started at metaphase, Cyclin B/Cdk1 decreases, Cdh1 is dephosphorylated, APC/C^{Cdh1} is activated and Ccd20 is degraded. At this stage of the cell cycle, APC^{Cdh1} takes over the degradation of mitotic cyclins, preventing their premature accumulation and premature entry into S-phase.

Schematic representation of regulation of activity of different APC/C complexes is shown in Figure 11.

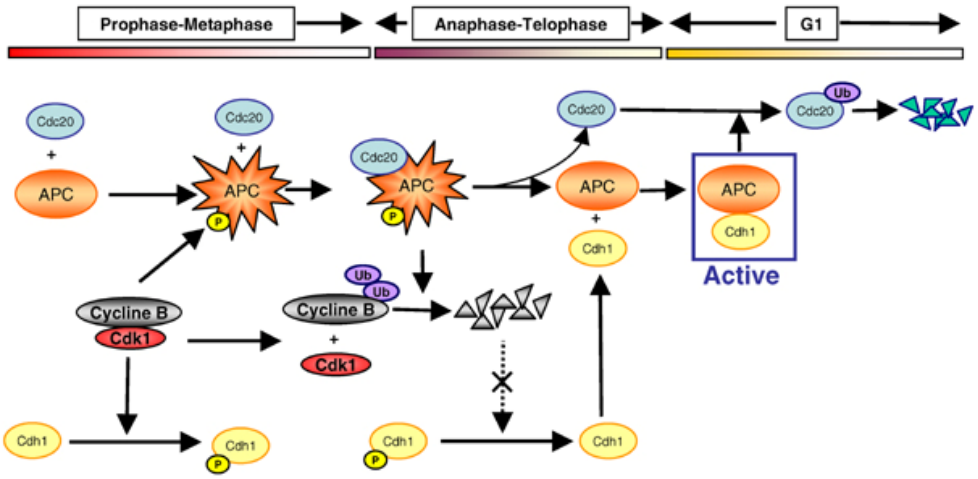


Figure 11.**Temporal pattern of APC/C^{cdc20} and APC/C^{Cdh1} regulation throughout the cell cycle.** (details in the text) (after Castro A. et al, 2005).

Besides Cdc20, Aurora A kinase is also degraded by APC/C during G1 phase. This proteolysis is exclusively mediated by APC/C^{Cdh1}, as I have described previously.

Phosphorylation of APC is one of the mechanisms used by cell to modulate its activity. The core subunits of APC: Apc1, Apc3/Cdc27, Apc6/Cdc16, Apc7 and Apc8/Cdc23, are phosphorylated during mitosis (Peters J.M. et al, 1996). This phosphorylation modulates Cdc20 binding to the APC and APC/C activity. Three different kinases have been described to phosphorylated APC: Cyclin B/Cdk1, Plk1 and PKA (Kotani S. et al, 1998), (Golan A. et al, 2002). Phosphorylation of core subunits by Cyclin B/Cdk1 increases Cdc20 binding and thereby APC/C activity (Kraft C. et al, 2003), whereas Plk1 phosphorylation does not influence neither Cdc20 binding nor APC/C activity. However, combination of both, Plk1 and Cyclin B/Cdk1 phosphorylation acts synergistically to increase APC/C ubiquitination abilities. *In vitro* phosphorylation of APC/C by PKA inhibits ubiquitination of Cyclin B even in the presence of regulatory factors Cdc20 and Cdh1 (Kotani S. et al, 1998).

Among negative regulators of APC/C on can find spindle checkpoint-dependent inhibitors, like Mad2, BubR1 and Mad2L2. Mad2 and BubR1 are known to inhibit APC/C^{Cdc20} (Fang G., 2002), whereas Mad2L2 was shown to specifically act on APC/C^{Cdh1}. Besides those, there are other two inhibiting proteins known: Emi1 (Hsu J.Y. et al, 2002), inhibiting both APC/C^{Cdc20} and APC/C^{Cdh1} by directly binding to the activators, and RASSF1 interacting exclusively with Cdc20 (Song M.S. et al, 2004).

2.6. Cell cycle checkpoints

Cell cycle checkpoints pathways mediate progression through the cell cycle and contribute to genomic stability, which is dependent on the fidelity of both DNA replication and chromosome segregation. Genomic stability is under constant threat from chemicals, radiation and normal cellular metabolism. Defects in the cell cycle checkpoint pathways are associated with an array of phenotypes in mammals – including cancer predisposition and neurodegeneration – consistent with the notion that checkpoint responses are critical for appropriate decision leading to cell survival or cell death.
Distinct checkpoints have been identified and characterized and the brief overview of them will be presented in the next chapters.

2.6.1. Spindle assembly checkpoint

The spindle assembly checkpoint delays sister chromatid separation until all chromosomes are properly aligned on the spindle. The checkpoint monitors the attachment of microtubules to kinetochores and generation of tension that results from bipolar attachment of sister chromatids.
Studies using mutant yeast strains have led to the identification of several kinases involved in spindle assembly checkpoint, namely Mps1, Bub1 and Bub3, as well as proteins Mad1, Mad2 and Mad3, which points to the important role of phosphorylation in the regulation of this checkpoint. According to our current understanding, structural changes, induced by microtubule attachment and tension, are translated, through phosphorylation, into a

biochemical signal. It has been proposed that interaction between the kinesin-related protein CENP-E and the kinase BubR1 is essential for this translation (Chan G.K. et al, 1999), (Yao X. et al, 2000), (Abrieu A. et al, 2000). How this regulates the kinetochore association of Mad proteins is unknown. However, unattached kinetochores are believed to function as sites of continuous assembly and release of Mad-Cdc20 complexes that prevent the activation of APC/C^{Cdc20}. On attachment of the last kinetochore, the production of inhibitory Mad2-Cdc20 complexes ceases, allowing Cdc20 to dissociate from Mad2 and activate APC/C/C. As a result securin is degraded and anaphase ensured.

Figure 13 represents schematic model of spindle assembly checkpoint.

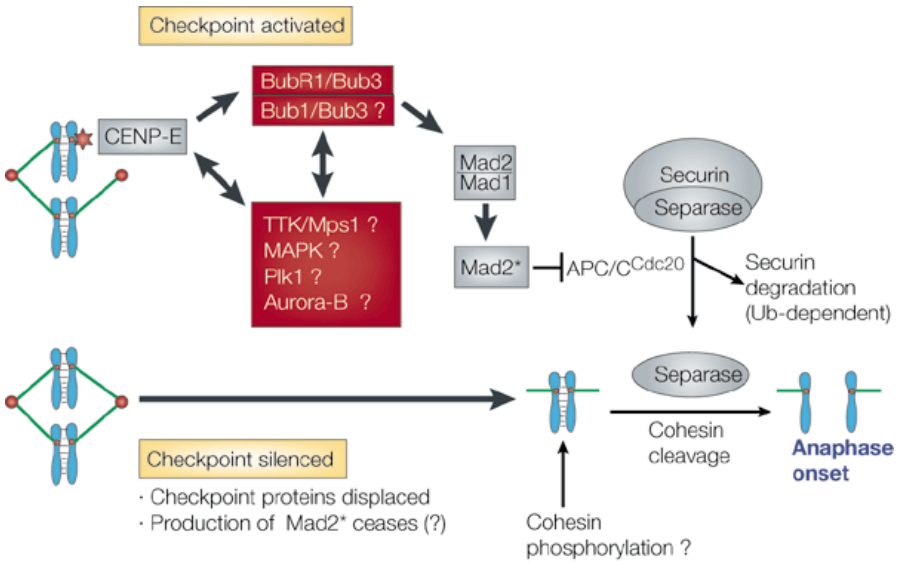


Figure 13. **The spindle assembly checkpoint.** One model holds that interaction between the kinesin-related protein CENP-E and BubR1 translate structural information (the presence or absence of appropriate microtubule-kinetochore interactions) into a chemical signal. These events regulate both recruitment of Mad1-Mad2 complexes to unattached kinetochores and the release of "conformationally altered Mad2" (represented by Mad2*). Mad2* then blocks a productive interaction between Cdc20 and APC/C, thereby preventing the degradation of securin and the cleavage of cohesin by separase. On attachment of the last kinetochore, the production of Mad2* drops, what ensues activation of APC/C^{Cdc20}. In addition to Bub1 and BubR1, activated MAP kinase (Mitogen Activated Kinase), Plk1 and Aurora B have been also detected at kinetochores, suggesting that these enzymes may function in either checkpoint signaling or silencing (after Nigg E.A., 2001).

At present it is not understood why mammalian cells express two Bub1 family members: Bub1 and BubR1. Both of them are required for checkpoint signaling (Chan G.K. et al, 1999), (Taylor S.S. and McKeon F., 1997), both are recruited to unattached kinetochores and they seem not to be redundant. Bub1 and BubR1 are recruited to the kinetochores in association with Bub3, a WD-repeat-containing substrate (Taylor S.S. et al, 1998), but what is the exact function – regulatory or effectory - of Bub3 is not known. Similarly the precise function of Mps1 family kinase needs to be resolved.

2.6.3. Spindle orientation (positioning) checkpoint

The spindle positioning checkpoint enforces the correct orientation of the elongation spindle to ensure that cleavage occurs in the right plane and only after complete separation of sister chromatids.

Evidences for the existence of spindle orientation checkpoint are coming from studies in budding yeast. Its silencing requires that a spindle pole body associates productively with the cortex of the budding cell, thus establishing a dependency between correct spindle positioning and mitotic exit (Hoyt M.A., 2000). The first identified component of this pathway was Bub2p, a spindle-pole-associated subunit of a two-component GTPase-activating protein (GAP). The latter down-regulates the activity of a small GTPase – Temp1p – that in turn functions at an early step in a pathway controlling mitotic exit. Downstream of active Temp1p, several kinases cooperate in so-called mitotic exit network (MEN) to activate Cdc14p phosphatase (Hoyt M.A., 2000). Cdc14p then acts not only as the activator of APC/C^{Cdh1}, but also dephosphorylates the Cdk1 inhibitor Sic1p, causing its stabilization, and the transcription factor Swi5p, which in turn enhances the Sic1p production. Therefore, inactivation of Cdk1 is ensured by three complementary mechanisms.

Gene products homologous to most components of the MEN pathway in *Saccharomyces cerevisiae* have also been found in fission yeast, although there is no evidence yet, that SIN (septation initiation network) plays a part in controlling Cdk1 inactivation. This would indicate that homologous gene products control partly distinct processes in two yeast.

The existence of spindle positioning checkpoint in organisms other than yeast still remains to be determined.

2.6.4. DNA structure/DNA damage checkpoints

The key components of mammalian DNA structure checkpoints' network can be divided into five categories, based on the position and general function in the network.

1. Sensors – among the plausible candidates for checkpoint sensors are the Rad9-Hus1-Rad1, so-called 9.1.1 complex, PCNA-like sliding clamp complex, the Rad17-RFC clamp loading complex and possibly the Mre11-Rad50-Nbs1 or MNR nuclease complex (Melo J. et al, 2002), (Petrini J.H. and Stracker T.H., 2003).
2. Mediators – include BRCA1, MDC1/NFBD1, 53BP1 and Claspin (Petrini J.H. and Stracker T.H., 2003).
3. Atypical signal transduction kinases – kinases of the phosphatidylinositol 3-kinase (PI3K)-like family, include ATM and ATR kinases (Abraham R.T., 2001), (Siloh Y., 2003).
4. Effector kinases – distal serine/threonine signal transduction kinases, represented by Chk1 and Chk2 (Bartek J. and Lucas J., 2003)
5. Effector proteins – large and diverse group encompassing cell cycle regulators such as Cdc25 phosphatase, various DNA repair proteins, transcription factors such as p53 and E2F, chromatin components and regulators such as histone H2AX and Tlk kinases, and others (Zhou B.B. and Elledge S.J., 2000), (Donzelli M. and Draetta G.F., 2003).

2.6.4.1. Checkpoint kinases

A central role for two groups of protein kinases, the ATM/ATR group and the Chk1/Chk2 group, in mediating the cellular responses to DNA damage has been established.

2.6.4.1.1. ATM/ATR

Both ATM (Ataxia Teleangiectasia Mutated) and ATR (ATM and Rad3-Related) belong to a group displaying homology to lipid kinases of the phosphatidylinositol-3-kinase (PI3-K) family. The yeast counterparts include Tel1 (*Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*), Mec1 (*S. cerevisiae*) and Rad3 (*S. pombe*). The importance of

this group of kinases in the response to DNA damage is emphasized by the fact that mutations in the human ATM gene are responsible for the recessive disorder ataxia teleangiectasia (AT), which results in progressive neurodegeneration, immune deficiency and the predisposition to cancer. These phenotypes all result from the inability of cells to properly handle damaged DNA (Jeggo P.A. et al, 1998).
The ATM kinase seems to primarily be activated following DNA damage whereas the ATR kinase seems to be critical for cellular responses to the arrest of DNA replication forks (Siloh Y. and Kastan M.B., 2001), (Abraham R.T, 2001). Many types of DNA damage, however, result both in the direct damage of the DNA and the arrest of DNA replication forks, therefore ATM and ATR seem to participate together in many cellular responses and complex joint response must be coordinated. Replication-fork arrest stimulates the initiation of cellular ATR activity, whereas DNA damage directly activates ATM and leads to replication-fork arrest, thereby also activating cellular ATR kinase.

ATM is an extremely large protein with predicted molecular mass of 350 kilodaltons. Patients, mice and cells lacking ATM are viable, suggesting that ATM kinase is not essential for critical cellular functions such as normal cell cycle progression or differentiation (Siloh Y. and Kastan M.B., 2001).
ATM kinase activity is minimal or low in unstressed cells and primarily is engaged to help cells deal with cellular stress that affect DNA or chromatin structure. ATM is present there as a homodimer in which the kinase domain is physically blocked by its tight binding to an internal domain of the protein surrounding serine 1981. The introduction of a DNA double strand break leads to a conformational change in the ATM protein, which stimulates the kinase to autophosphorylate serine 1981, causing the dissociation of the homodimer (Bakkenist C.J. and Kastan M.B., 2003). The activated monomer is now able to phosphorylate its numerous substrates: p53, NBS1 (Nijemegen breakage syndrome 1), BRCA1 (breast cancer 1) or SMC1 (structural maintenance of chromosome 1). The conformational change, which induces extremely rapid autophosphorylation of ATM, does not seem to require the binding of ATM to the site of DNA damage, but instead results from some change in higher-order chromatin structure that the ATM dimer can sense at some distance away from the site of the DNA break. The exact nature of this "sensing" is, however, not known. Recent observations postulate a role of MRE11/RAD50/NBS1 complex in the activation of ATM upon ionizing radiation (Uziel T. et al, 2003), (Carson C.T. et al, 2003). This role was shown to be mainly binding to ATM and helping its

translocation to the sites of DNA damage (Lee J.H. and Paull T.T., 2004). Once recruited to the DNA break, the activated ATM can then phosphorylate critical substrates like NBS1, BRCA1 and SMC1, which accumulate at these sites. If ATM is activated, but fails to translocate to the site of the DNA break (in the absence of NBS1 or BRCA1) it can still phosphorylate its nucleoplasmic substrates such as p53.

ATR is a large protein, with a predicted molecular mass of 301 kilodaltons. Unlike in the case of ATM, cell and animals lacking ATR seem to be non-viable (Brown E.J. and Baltimore D., 2003). These observations suggest that ATR is required for normal progression through the cell cycle, even in the absence of cellular stress. ATR has been reported to play a role in normal progression of DNA replication forks (Shechter D. et al, 2004) as well as in other types of cellular stress, like hypoxia (Hammond E.M. et al, 2002) and in cellular response to DNA-replication inhibitors (Hekmat-Nejad M. et al, 2000). Finally, ATR seems to be engaged in the response to DNA breaks, possibly compensating for ATM, since many ATM substrates get phosphorylated after induction of double strand breaks in cells lacking ATM proteins (Krystyniak A. unpublished results).
It seems that ATR kinase may be constitutively ready to phosphorylate substrates but have its cellular function controlled by sub-cellular localization. ATR exists, independently on the exposure to stress, in a complex with the ATR-interacting protein (ATRIP) (Cortez D. et al, 2001). The observation has been made that replication protein A (RPA), a single-stranded DNA-binding protein involved in DNA replication, stimulates the *in vitro* binding of ATRIP to ssDNA. This has led to a model in which ATR becomes localized to sites of replication fork arrest by means of binding of ATRIP to RPA (Zou L. and Elledge S.J., 2003). Any stimulation or stress that leads to an abnormal stretch of single stranded DNA, such as an arrested replication fork, would be decorated with RPA. The accumulation of RPA would then lead to the recruitment of ATRIP protein together with its binding partner ATR. Once active ATR kinase is localized to the single stranded DNA region, it can phosphorylate its critical substrates, such as RAD17 and Chk1. As with ATM, the presence of an active ATR kinase in the cells is not sufficient for ATR to carry its cellular functions. In addition to ATR, several other proteins must be recruited to the sites of ssDNA as well. These include the clamp loading Rad17-RFC complex, RSR, which participates in the loading of the Rad9-Hus1-Rad1 sliding clamp into chromatin, and the claspin protein, which is independently recruited to chromatin (Ellison V. and Stillman B., 2001), (Osborn

A.J. et al, 2002). All these events are required for the phosphorylation of Chk1 by ATR and for the activation of the appropriate cell cycle checkpoint.

Figure 14 presents a model of ATM and ATR activation.

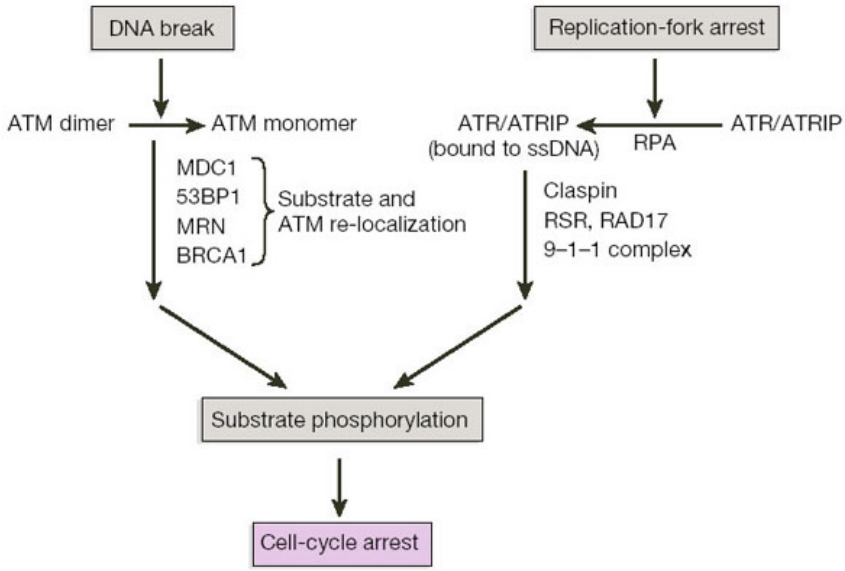


Figure 14. **Scheme of mechanisms that lead to the induction of ATM- and ATR-directed cellular activities.** DNA strand breaks lead to the dissociation of the inactive ATM dimer. The appropriate localization of both the ATM monomer and the ATM substrates is modulated by several proteins, including the MRN complex, MDC1, 53BP1 and BRCA1. The ATR/ATRIP complex is recruited to sites of ssDNA, perhaps by RPA. Optimal substrate phosphorylation and the engagement of cell cycle arrest depends on other proteins such as claspins, the RSR complex and the 9-1-1 complex.

2.6.4.1.2. Chk1/Chk2

The second group of kinases consists of serine/threonine kinases Chk1 and Chk2. Chk1 was first identified in *Schizosaccharomyces pombe* and shown to be required for cell cycle arrest in response to DNA damage. Homologues have been found in *S. cerevisiae*, mammals, *Xenopus* and flies (O'Connell M.J., et al, 2000). Chk2 is the mammalian homologue of Cds1 in *S. pombe* and *Xenopus* and Rad53 in *S. cerevisiae*. Mutations in

human Chk2 have been uncovered in patients predisposed to cancer with Li-Fraumeni syndrome (LFS) (Bell D.W. et al, 1999).

Chk2 is a relatively stable protein, with a half-life longer than six hours, which is expressed and can be activated in all phases of the cell cycle. As mentioned above, several kinds of genotoxic stresses can activate the ATM kinase, which in turn phosphorylates the N-terminal regulatory domain of Chk2, at many sites, the most prominent being threonine 68 (Bartek J. et al., 2001). This in turn promotes homodimerization and intermolecular transphosphorylation of Chk2 on its C-terminal kinase domain (Ahn J.Y. et al., 2002), (Xu X. et al., 2002), (Lee C.H. and Chung J.H., 2001), a modification required for a full activation of Chk2 toward heterologous substrates (Figure 15).

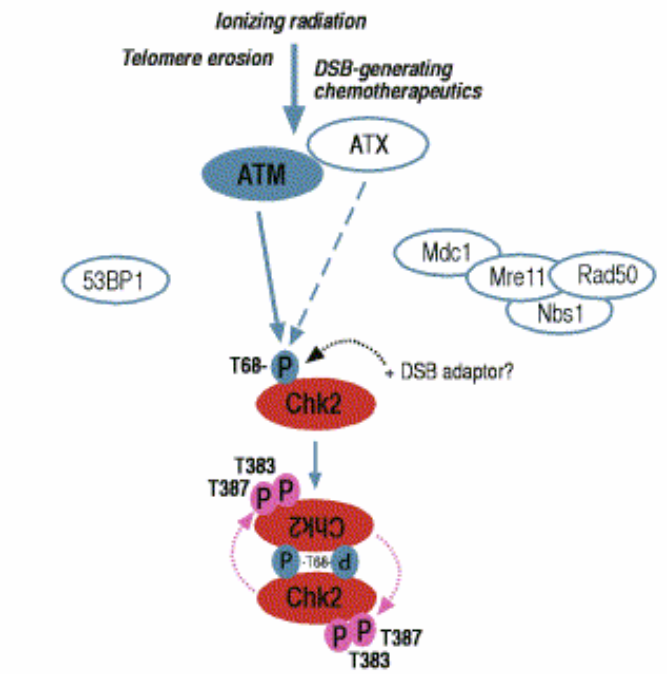


Figure 15. **Regulation of Chk2 activity by phosphorylation.** (by Bartek J. and Lukas J., 2003).

A recent report indicates that the ATM-dependent phosphorylation of Chk2 cannot occur freely in the nucleoplasm, but requires a specific DSB-associated adaptor protein(s) (Lukas J. et al., 2003). In addition, other checkpoint proteins may co-regulate the physiological velocity and/or timing of Chk2 activation. These factors include a DSB-interacting protein (53BP1), DNA ends-processing MRN nuclease complex (Mre11/Rad50/Nbs1), and its newly identified binding partner Mdc1.

By contrast to Chk2, Chk1 protein is unstable, with a half-life of less than two hours and its expression is restricted to S and G2 phases of the cell cycle. Activation of ATR, by mainly stalled replication, induces phosphorylation of its main substrate Chk1 (Feijoo C. et al., 2001), (Heffernan T.P. et al., 2002), (Shiloh Y., 2003). Several recent studies show that ATM can also phosphorylate Chk1 in cells exposed to IR, although to a lesser extent compared to the ATR-mediated effect after other types of DNA damage (Gatei M. et al., 2003). Both ATR and ATM target the SQ-rich C terminus of Chk1, including serines 317 and 345, respectively. These phosphorylations may directly lead to Chk1 activation. Optimal activation of Chk1 also requires a cooperative action of other factors including the multifunctional BRCA1 tumor suppressor (Yarden R.I. et al., 2002), the claspin adaptor molecule, and the PCNA-like DNA sliding clamp (Rad9/Rad1/Hus1) together with its loading factor (Rad17) (Figure 17).

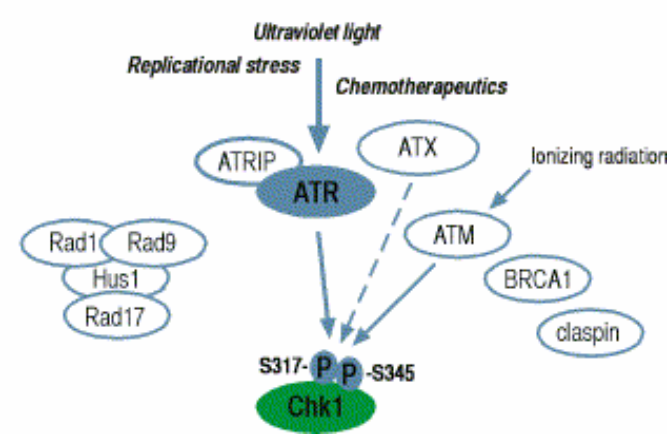


Figure 16. **Regulation of Chk1 activity.** (by Bartek J. and Lukas J., 2003).

Following their activation, Chk1 and Chk2 phosphorylate unique and overlapping downstream effectors that further propagate the checkpoint signaling. Depending on the type of stress, extent of DNA damage, and cellular context, this leads to switch to the stress-induced transcription program (E2F1, BRCA1, p53), direct or indirect initiation of DNA repair (BRCA1, p53), acute delay (degradation of Cdc25A) and/or sustained block (Cdc25C, p53, Plk3) of cell cycle progression, apoptosis (Pml1, p53, E2F1), and modulation of the chromatin remodeling pathways (Tlk1/2).

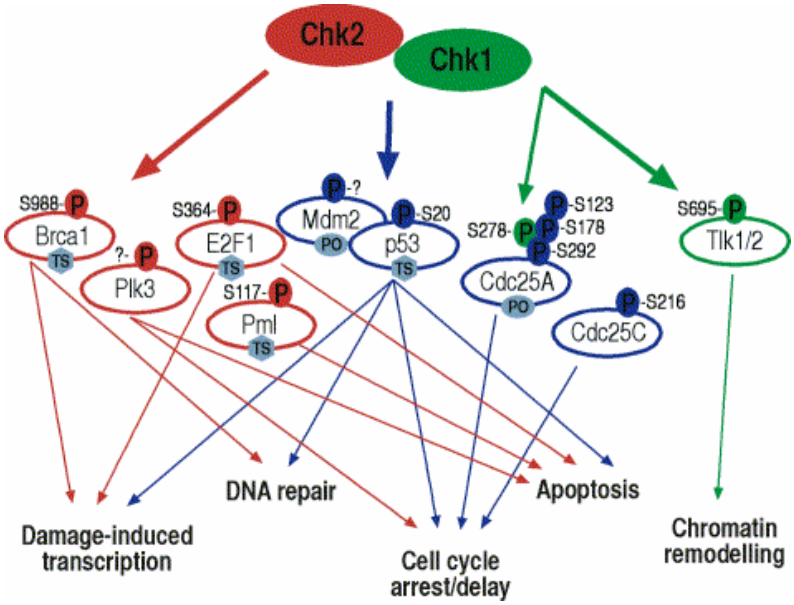


Figure 17. **Chk1 and Chk2 as mediators of the checkpoint-signaling network.** In red unique Chk2 substrates, in green unique Chk1 substrates, in blue – overlapping ones. The known target sites of Chk1 (green), Chk2 (red), and both Chk1 and Chk2 (blue) on the individual substrates are shown. Some of the Chk1/Chk2 downstream effectors are classified as protooncogenes (PO) or tumor suppressors (TS), as indicated (by Bartek J. and Lukas J., 2003).

2.6.4.2. G1 checkpoint

To prevent entry into S-phase with damaged DNA, cells traversing G1 activate the checkpoint transducing kinases ATM/ATR and Chk1/Chk2 which, in turn, target two critical effectors operating in distinct branches of the G1 checkpoint, the Cdc25 phosphatase and the transcription factor p53 (Lukas J. et al, 2004).

The phosphorylation of Cdc25A on multiple serine residues by Chk1 and Chk2 leads to its enhanced ubiquitination and degradation (Zhao H. et al, 2002), (Sorensen C.S. et al, 2003), by $SCF^{\beta TRCP}$ (described earlier), thereby preventing the Cdc25A-mediated activatory dephosphorylation of Cdk2 (Mailand N. et al, 2000). Such inhibition of Cdk2 blocks loading onto chromatin of Cdc45, a protein essential for recruitment of DNA polymerase α into assembled pre-replication complexes, thus preventing DNA synthesis (Falck J. et al, 2002). The checkpoint pathway targeting Cdc25 A is implemented rapidly, operates independently on the p53 status and is relatively transient, capable of delaying cell cycle progression only for several hours (Mainland N. et al, 2000). On the other hand, the

complementary mechanism responsible for prolonged maintenance of the G1 cell cycle arrest in response to DNA damage, depends on p53.

In contrast to Cdc25A, p53 phosphorylation is carried out not only by Chk1 and Chk2, phosphorylating p53 on threonine 18 and serine 20 (and possibly other residues), but also directly by the upstream checkpoint kinases ATM and ATR, particularly on serine 15. Additionally, the ubiquitin ligase Mdm2, responsible for p53 degradation is also targeted by ATM/ATR in response to DNA damage. The detailed mechanism of p53 involvement in the cell cycle arrest is described in chapter 2.6.5. Accumulation of p21, p53 target, sufficient for blocking G1/S promoting cyclins may require up to several hours, and this mechanism complements and eventually replaces the transient acute inhibition of Cdk2 through the Cdc25A degradation pathway, thereby leading to a sustained, or sometimes permanent cell cycle blockade. Figure 18 shows schematically the order of events leading to G1/S arrest.

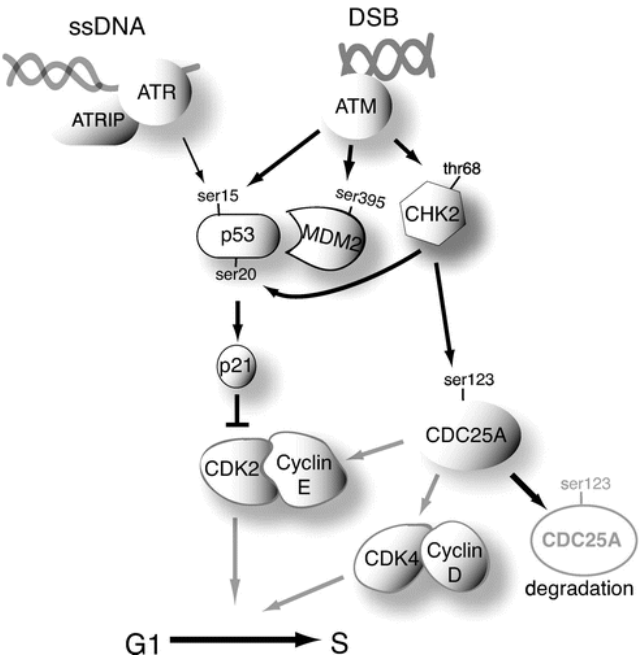


Figure 18. **G1/S checkpoint.**
(after Nyberg K.A. et el, 2002)
(description in the text)

2.6.4.3. Intra S-phase checkpoint

The S-phase checkpoint activated by genotoxic insults causes only transient, reversible delay in cell cycle progression, mainly by inhibition of new replicon initiation, thereby

resulting in a slow down of DNA replication. Thus, unlike the G1 and G2/M checkpoints, the intra-S-phase response to DNA damage lack sustained cell cycle arrest.

It seems that there are at least two parallel pathways in this checkpoint to slow down ongoing DNA synthesis, both of them controlled by the ATM/ATR machinery. One of these effector mechanisms operates via the Cdc25A cascade, described in the previous chapter. The other pathway reflects the impact of ATM-mediated phosphorylation of NBS1 on several sites, in particular serine 343 (Siloh Y., 2003) as well as serine 957 and 966 of the cohesin protein, SMC1 (Kim S.T. et al, 2002), (Kitagawa R. et al, 2004). Some role of proteins BRCA1 and FANCD2 (Fanconi anaemia, complementation group 2) in mediating this action has been reported (Taniguchi T. et al, 2002), (Nakanishi K. et al, 2002).

Most recently, the S-phase kinases Cdc7/Dbf4 (Costanzo V. et al, 2003) and the Tlk1 (Groth A. et al, 2003), (Krause D.R. et al, 2003) have also turned out to be targeted by the checkpoint kinase ATM/ATR and Chk1, although the exact mechanism and the biological significance of these events remain unknown.

Apart from the inhibition of replication-origin firing, another critical function provided by S-phase checkpoint is to protect the integrity of the stalled replication forks. Such maintenance of fork stability, achieved through yet unknown mechanism, helps prevent the conversion of primary lesions into DNA breaks and facilitates the subsequent recovery of DNA replication (Bartek J. et al, 2004).

2.6.4.4. G2 (G2/M) checkpoint

The G2 checkpoint prevents cells form entering mitosis when DNA is damaged, either as a result of damaged experienced during G2 or when cells progressed into G2 with some unrepaired damage inflicted during previous S or G1 phases. G2 accumulation may also result from so-called DNA-replication checkpoint or S/M checkpoint that may sense some of the persistent lesions from the previous S-phase as being inappropriately or not fully replicated DNA.

The critical target of the G2 checkpoint is the mitosis-promoting activity of the Cyclin B/Cdk1 kinase. Upon DNA damage Cyclin B/Cdk1 activation is inhibited by ATM/ATR and Chk1/Chk2-mediated sub-cellular sequestration as well as degradation and inhibition of the Cdc25 family of phosphatases that normally activate Cdk1 at the G2/M boundary

(Mailand N. et al, 2002), (Donzelli M. and Draetta G.F., 2003). Lately, it turned out that all three members of the Cdc25 family of phosphatases Cdc25A, Cdc25B and Cdc25C cooperate as positive regulators of Cyclin B/Cdk1 in unperturbed cell cycle. In response to DNA damage or incompletely replicated DNA, Ccd25A becomes degraded in G2, most likely via the same mechanism, that has been described above for the G1 and S-phase checkpoints (Xiao Z. et al, 2003). The Cdc25C phosphatase activity is instead blocked by its binding to 14-3-3 protein, a process induced by phosphorylation of Cdc25C on serine 216 by kinases Chk1 and Chk2. The role of Cdc25B in G2 checkpoint has been connected with its phosphorylation at serine 309 by MAP kinase p38, which also induces binding to 14-3-3 (Bulavin D.V. et al, 2001).

Another level of complexity is added by the fact that other upstream regulators of Cdc25C and/or Cyclin B/Cdk1, such as the Polo like kinases, Plk1 and Plk3, seem to be targeted by DNA damage-induced mechanism. Scheme of G2 checkpoint is presented in Figure 19.

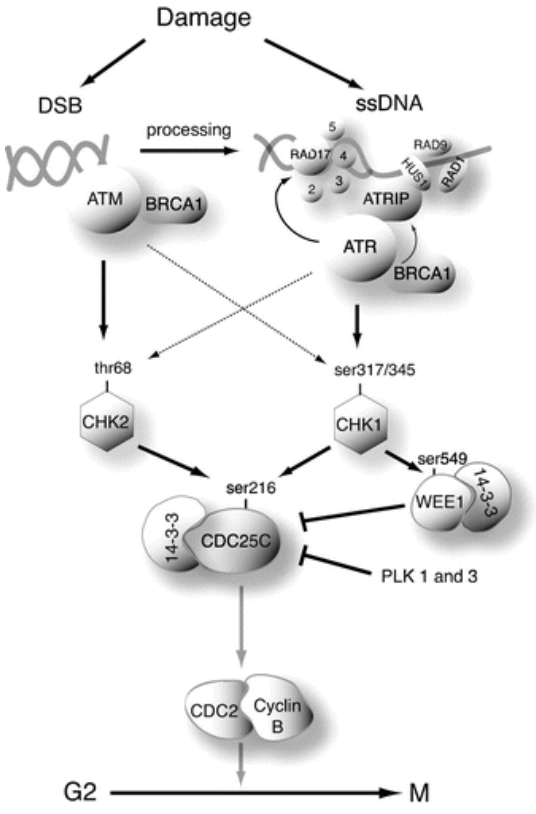


Figure 19. **The G2/M checkpoint.**
(after Nyberg K.A. et el, 2002) (description in the text)

One mechanism that contributes to long-term silencing of Cyclin B/Cdk1 is through p53 pathway, described in the next chapter. On the other hand, many cell types lacking p53 still tend to accumulate in G2 after DNA damage, indicating that additional mechanisms, such

as the BRCA1-stimulated expression of p21 and GADD45 (Nyberg K.A. et al, 2002) may cooperate with the p53 cascade in regulating the delayed, sustained G2-arrest.

2.6.5. p53 as a target of multiple checkpoint pathways

The p53 tumor suppressor belongs to a small family of related proteins, that include two other members: p63 and p73 (Vousden K.H. and Lu X., 2002). Although structurally and functionally related, p63 and p73 have roles in normal development, whereas p53 seems to have evolved in higher organisms to prevent tumor development. Several responses can be provoked by p53, including cell cycle arrest, apoptosis, senescence, cell differentiation and angiogenesis (Sionov R.V. and Haupt Y., 1999).

p53 plays a role in regulation various checkpoints during the cell cycle. The induction of $p21^{WAF/Cip1}$ is responsible for G1 arrest, while the induction of 14-3-3σ, and to some extent that of GADD45 gene, mediate the G2 arrest. These checkpoints prevent the cell with damaged DNA from undergoing cell cycle progression (replication or mitosis, respectively).

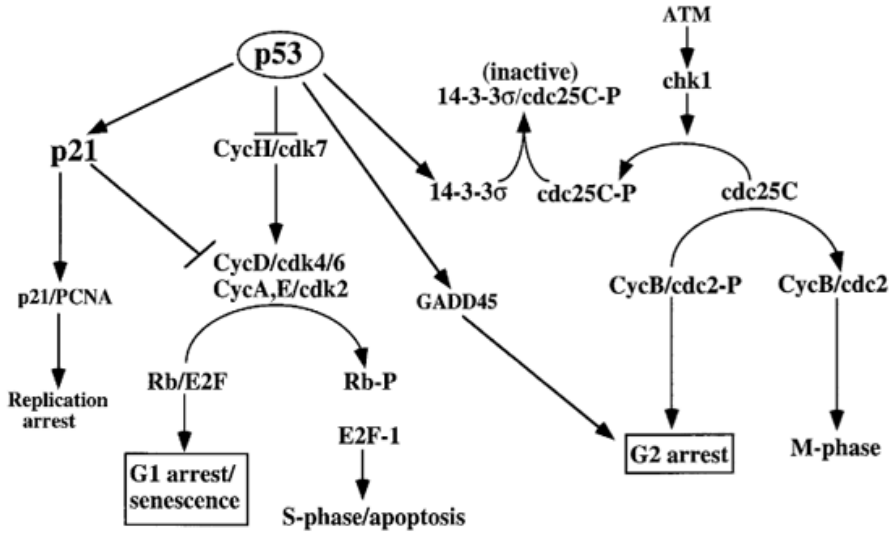


Figure 20. **Induction of growth arrest by p53.** Activation of p53 induces the transcription of target genes involved in cell cycle regulation. P21 is central for the induction of G1 arrest/senescence and replication arrest. GADD45 and 14-3-3σ promote G2 arrest through separate pathways. The parallel contribution of ATM is also included. (after Sionov R.V. and Haupt Y., 1999).

p21 mediates p53-dependent G1 arrest by inhibiting the activity of Cdks though its N-terminal domain, leading to failure of Rb phosphorylation (as described in chapter 2.1.1.1). In addition interaction of p21 C-terminal domain with PCNA prevents activation of DNA polymerase δ, what leads to replication arrest.
p53 can also contribute to block of the cell cycle independently of p21. By binding to Cyclin H and $p36^{MAT1}$, p53 inhibits CAK, thereby preventing the activation of Cdk2/Cyclin A and blocking the G1/S transition.

In addition, p53 was also shown to efficiently induce G2 arrest. The p53-induced gene product - 14-3-3σ sequesters the phosphorylated form of cdc25C, thereby preventing G2/M transition. In addition, another p53 target – GADD45 disrupts the CyclinB/Cdk1 complex, probably via interaction with Cdk1, leading to inhibition of kinase activity and arrest at G2/M. Although G2 arrest can occur in the absence of p21 or p53, both proteins are essential for sustaining the G2 arrest after DNA damage (Bunz F. et al, 1998).

The p53 protein has a very short half-life in normal condition, however exposure of cells to DNA damage leads to rapid accumulation of the protein. The mechanism of p53 stabilization involves release of the p53 binding protein Mdm2 (Murine double minute 2), a proto-oncogene displaying E3 ligase activity and responsible for ubiquitinylation-dependent p53 degradation. In an unperturbed cell cycle Mdm2 binds to p53 within its transactivation domain, blocks its transcriptional activity, and abrogates p53 ability to stop the cell cycle and induce apoptosis. Since the Mdm2 gene is direct target of p53, a negative autoregulatory feedback loop exists between those two proteins.
It was known that DNA damage destabilizes Mdm2 by mechanism involving damage-activated kinases and Mdm2 auto-ubiquitination, thereby preventing p53 degradation and stabilizing it (Stommel J.M. and Wahl G.M., 2004).

3. AURORA A KINASE

3.1. Aurora kinase family

Aurora is the name given to a family of serine/threonine protein kinases that regulate many aspects of cell division. They are known to be involved in the control of centrosome and nuclear cycles, and have essential functions in mitotic processes, such as chromosome condensation, spindle dynamics, kinetochore-microtuble interactions, chromosome orientation and establishment of the metaphase plate as well as the proper completion of cytokinesis (Carmena M. and Earnshaw W.C., 2003).
The original aurora allele was identified in a screen for *Drosophila melanogaster* mutants defective in spindle-pole behavior, and was named after the phenomenon of the night sky in the polar regions (Glover D.M. et al, 1995) The first member of the family was however identified in *Xenopus* (named Eg2) (Paris J. and Philippe M., 1990). We know now that there are three types of Aurora in mammals (Aurora A, Aurora B and Aurora C), two in frogs, *Drosophila* and *C.elegans* (the A- and B-types), and a single one in budding (Ipl1) and fission (Ark1) yeast which seem to be most similar to B-type in function.

Human Auroras A-C are kinases of a size ranging from 309 to 403 amino acid residues that exhibit a relatively high sequence divergence between species. As shown in Figure 21 they show similar domain organization: a N-terminal domain of 39-129 residues in length, a protein kinase domain and a short C-terminal domain of 15-20 residues. The N-terminal domain displays relatively low sequence conservation, within members of the family and this reflects on the selectivity of protein-protein interaction.
The catalytic domain, on the other hand, is more conserved. The C-terminal domain of Aurora B shares 53% and 73% sequence similarity to human Auroras A and C, respectively. The comparison of the crystal structure of human Aurora A against the predicted structure of Aurora B and C also supports the thesis that vertebrate Aurora B and C are closely related paralogs (Brown J.K. et al, 2004).
The alignment of Aurora A and B pointed to the presence of a distantly conserved KEN motif, spanning 11-18 residues, which acts as Cdh1-dependent APC/C recognition signal.

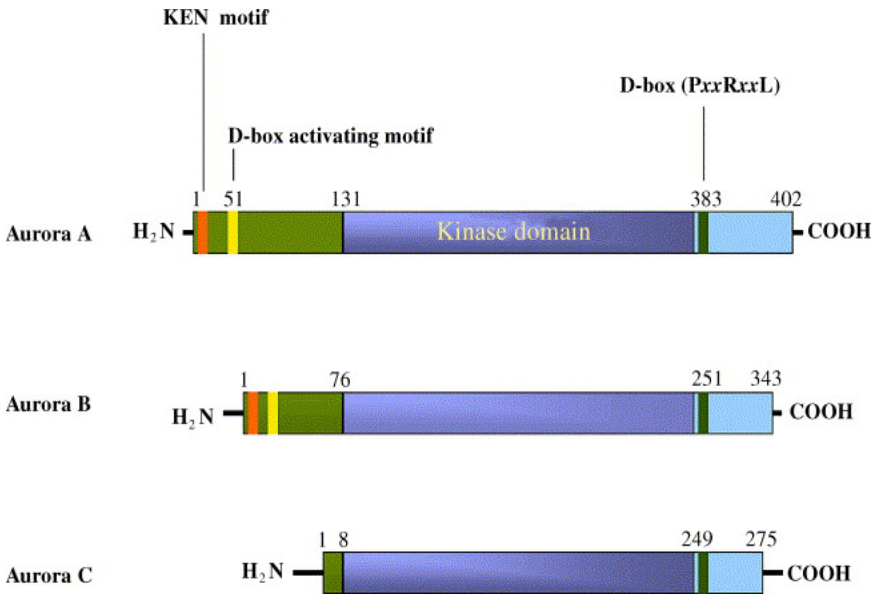


Figure 21**. Domain organization of Aurora kinases A–C**. As shown here, Aurora kinases present three domains: The N- and the C-terminal domains contain most of the Aurora's regulatory motifs, while the central region contains the catalytic domain. In addition to the kinase activity, this central domain also presents regulatory motifs, as the crystal structure of the Aurora A-TPX2 complex has shown (after Bolans-Garcia V.M., 2005)

Surprisingly, given this level of similarity, the three mammalian Aurora kinases have very distinct localizations and functions.

Aurora A kinase is associated with the centrosome from the time of centrosome duplication to mitotic exit and with regions of microtubules proximal to centrosomes in mitosis. Aurora B forms a complex with two proteins, inner centromere protein (INCENP) and survivin and behaves like a chromosomal "passenger" protein. Less is known about Aurora C kinase, which is specifically expressed at high levels in the testis and shows centrosomal localization from anaphase to telophase.

Despite the high conservation of their catalytic domains, which would indicate similar site specificity, Aurora kinases differ in the substrate specificity. This is likely explained by the different sub-cellular localization of Aurora family members and by the interaction with different sets of partners. A brief overview of specific Auroras' substrates, their cellular localization and function is presented in Table 3.

	Substrate	Cell localization	Effect of substrates-Aurora interaction
AurA	PP1 p53 Cdh-1 TPX-2 RasGAP Ajuba	mitotic spindle centrosome	spindle assembly cytokinesis centrosome maturation and separation
AurB	histone H3 INCENP CENP-A desmin Rec-8 vimentin; MCAK; survivin	centrosome central spindles chromosome arms	chromosome alignment and segregation cytokinesis microtubule dynamics
AurC	Aurora B INCENP.	central spindles chromosome arms?	role in spermatogenesis possible role in regulation of chromosome segregation and cytokinesis

Table 3. **Human Aurora kinases A–C exhibit differential substrate affinity, subcell localization and associated activities** (after Bolans-Garcia V.M., 2005)

I will now describe each of the Aurora family members in more details, taking the "alphabetically-reverse" order, which corresponds more logically to the aim of my study.

3.1.1. Aurora C

Aurora C was first identified in a screen for kinases expressed in mouse sperm and eggs (Tseng T.C. et al, 1998) and for long time was believed to function only in testis. Only later Aurora C over-expression was also demonstrated in certain cancer cell lines (Kimura M. et al, 1999). The Aurora C gene is localized to 19q13.43 region, which is often translocated or deleted in certain cancer tissues. Its protein level seems to be cell cycle regulated with low expression in interphase and peak levels at G2 and mitosis. Aurora C localizes to centrosomes in anaphase and persists there until cytokinesis, suggesting that it may have a role during late stages of M-phase (Kimura M. et al, 1999). Little is known about regulation of protein and kinase activity, with the exception of a report an

apparently negative phosphorylation site (T171), which is recognized and phosphorylated *in vitro* by protein kinase A (PKA) (Chen S.S. et al, 2002). Aurora C was found to be activated by INCENP (inner centromere protein), and over-expression of those two proteins was shown to increase phosphorylation of histone H3, an event that is rather under the control of Aurora B (see chapter 3.1.2) (Li X. et al, 2004), (Sasai K. et al, 2004).

3.1.2. Aurora B

Human Aurora B was identified in a PCR screening for kinases overexpressed in colorectal cancers (Bischoff J.R. et al, 1998) and it was later shown to be involved in several important processes during mitosis.

Aurora B is localized along the length of chromosomes at prophase, whereas it relocates at the inner centromere regions near the kinetochores during prometaphase and metaphase. At anaphase it is present at the spindle midzone and finally in the midbody at the end of cytokinesis (Adams R.R. et al, 2001).

Aurora B protein and activity levels are tightly regulated during transition through the cell cycle, with a peak of protein expression at the G2/M transition and the maximal activity at mitosis. The activity of Aurora B is regulated based on two independent mechanisms: phosphorylation and protein-protein interaction. It has been shown that Aurora B kinase activity dramatically increases upon treatment with the phosphatase inhibitor, okadaic acid, yet the kinase responsible for phosphorylation of Aurora B has not been found (Sugiyama K. et al, 2002). The same study has also shown that Aurora B physically associates with protein serine/threonine phosphatase type 1 (PP1) and PP2A that likely contribute to determine the balance between positive and negative inputs affecting Aurora B activity. Moreover, Aurora B activity is positively regulated through the association INCENP and survivin. The former is a microtubule-binding protein that is targeted to the chromosomes and centromeres through its non-conserved N-terminal region. INCENP contains a motif (IN-box) in its highly conserved C-terminal region that is responsible for binding to Aurora. (Honda R. et al, 2003). Aurora B-dependent phosphorylation of a site contained the IN-box functions in a positive feedback loop to enhance Aurora activity (Bishop J.D and Schumacher J.M., 2002). Survivin is a small protein that contains a BIR (baculovirus inhibitor of apoptosis (IAP) repeat) domain and a Zinc-finger motif typical for IAP proteins. Survivin was shown to enhance Aurora B kinase activity *in vitro* (Bolton M.A. et

al. 2002), and treatment with siRNA against survivin resulted in decrease of Aurora B activity and mislocalization of the kinase (Chen J. et al, 2003).

Aurora B kinase is responsible for phosphorylation of histone H3 on serine 10 (Hsu J.Y. et al, 2000), (Giet R. and Glover D.M., 2001), (Crosio C. et al, 2002) a post-translational modification that is associated with chromatin condensation. It was also found to be responsible for phosphorylation of H3 at serine 28 from prophase to metaphase (Goto H. et al, 2002). Other specific substrates of Aurora B are myosine II regulatory light chain (Murata-Hori M. et al, 2000), vimentin (Goto H. et al, 2003), desmin and glial fibrillary acidic protein (GFAP) (Kawajiri A. et al, 2003), all of them involved in late stages of cytokinesis. Also MgcRacGAP, a known activator of Rho A important for actin polymerization and completion of cytokinesis, was found to be phosphorylated by Aurora B (Jantsch-Plunger V. et al, 2000). Some role in meiosis, namely phosphorylation of Rec8, a meiotic-specific subunit of the cohesin complex was also attributed to Aurora B (Rogers E. et al, 2002).

The role of Aurora B in DNA damage response has been postulated recently. Aurora B was shown to physically and specifically associate with the BRCT (BRCA-1 C-terminal) domain of PARP-1 (poly(ADP-ribose) polymerase 1), a protein involved in DNA damage detection and repair. In response to DNA damage Aurora B becomes highly poly(ADP-ribosyl)ated, an event that leads to the inhibition of its kinase activity and hence to reduction of serine 10 phosphorylation of histone H3 (Monaco L. et al, 2005)

3.2. Aurora A

The gene encoding Aurora A is located on chromosome 20, in a region often amplified in human tumors (20q13). Overexpression of Aurora A protein has been reported in several cancers, such as breast (Royce M.E. et al, 2003), colorectal (Bischoff J.R. et al, 1998), bladder (Tseng Y-S. et al, 2005), thyroid (Ulisse S. et al, 2006), NHL (Non-Hodgkin's Lymphoma) (Yakushijin Y. et al, 2004) and gastric cancer (Yamada K.K. et al, 2004) in a manner that is both dependent and independent from gene amplification.

The human gene exhibits significant homology with the previously cloned prototypic yeast Ipl1 (40% of identity) and *Drosophila* aurora (48% of identity) protein serine/threonine kinase-encoding genes. The translated human Aurora A protein consists of 403 amino acids and has a molecular weight of 46 kilodaltons (Sens S. et al, 1997).

Aurora A has an important regulatory role in spindle formation, and therefore is essential for accurate chromosome segregation. Depending on the organism and on differences in their cell cycle, Aurora A mutations interfere with recruitment of γ-tubulin ring complex and other proteins during centrosome maturation, formation of a bipolar spindle, maintenance of the bipolar spindle, and chromosome segregation (Crane R. et al, 2003).

3.2.1. AurA regulation

Aurora A level as well as its activity is tightly cell cycle regulated, being low at G1 and S, increasing at G2, reaching the maximum at mitosis and then being rapidly degraded (Bischoff J.R. et al, 1998), (Krystyniak A et al, 2006).

3.2.1.1. Phosphorylation

Similarly to other Aurora family members Aurora A activity is regulated by phosphorylation. The phosphorylation of a conserved residue, threonine 288, localized in the activation loop of the catalytic domain of the kinase, results in a significant increase of Aurora A kinase activity. Threonine 288 can be phosphorylated *in vitro* by protein kinase A (PKA) (Walter A.O. et al, 2000). However a role for PKA *in vivo* is questionable since PKA activity is low when cells approach mitosis and Aurora A can autophosphorylate at a number of sites, including threonine 288 and thereby autoactivate (Ferrari S. et al, 2005). Two other phosphorylation sites have been found by mass spectroscopy at mitosis in *Xenopus* Aurora A, namely serine 53 and serine 349 (S51 and S342 in the human Aurora A) (Littlepage L.E. et al, 2002). S53 is part of the A-box, a highly conserved motif required for destruction of Aurora A at mitosis, whereas S349 is N-terminal to a protein phosphatase 1 (PP1)-binding motif. Mutations of serine 53 to both alanine and aspartic acid had no significant effect on kinase activity. However, the Asp53 was shown to be more stable than wild type, suggesting that phosphorylation at this site might regulate Aurora A destruction during mitosis (Littlepage L.E. et al, 2002). Mutation of serine 349 to alanine slightly reduced Aurora A activity, whereas mutation of this site to aspartic acid completely abolished it. The authors suggested that either phosphorylation of serine 342 is inhibitory, or that it is somehow critical for the conformation of Aurora A in some other

way. Our data indicated that mutation of S342 to alanine in human Aurora A resulted in an isoform displaying the same activity as wild type. Therefore, given its close proximity to the PP1 binding motif, it is more likely that phosphorylation at S342 is involved in the control of the interaction of Aurora A with critical regulators (Krystyniak A. et al, manuscript in preparation).

3.2.1.2. Binding partners

3.2.1.2.1. Activation of Aurora A by TPX2

TPX2 (Target Protein for *Xenopus* kinesin-like protein 2) is a microtubule-associated protein, which is required for induction of spindle assembly (Gruss O.J. et al, 2002). TPX2 appears to be tightly regulated during the cell cycle, with protein level peaking at mitosis and declining sharply during mitotic exit (Steward S. and Fang G., 2005). TPX2 is a key target of the Ran-GTP pathway. It was found to be sequestered through its association with importin β in the absence of Ran-GTP, whereas during mitosis, Ran-GTP, that is generated by RCC1 around chromosomes disrupts the TPX2 - importin β complex, thus releasing active TPX2 to initiate bipolar spindle assembly (Gruss O.J. at al, 2002). Similarly, human TPX2 is involved in spindle formation, spindle pole organization and centrosome integrity, as shown in experiments with siRNA (Garret S. et al, 2002).
It has been shown that N-terminus of TPX2 binds to C-terminal catalytic domain of Aurora A, and targets it to the spindle apparatus (Kufer T.A. et al, 2002). Upon siRNA-mediated depletion of TPX2, the association of Aurora A with spindle microtubules was abolished, although its association with spindle poles was not affected. Depletion of Aurora A, on the other hand, did not result in mislocalization of TPX2. Further studies have shown that *in vitro*-dephosphorylated Aurora A, becomes phosphorylated and activated by TPX2 in an ATP dependent manner (Eyers P.A. et al, 2003). Increased phosphorylation and activation of Aurora A requires its own kinase activity, suggesting that TPX2 stimulates autophosphorylation and autoactivation of the enzyme. The molecular mechanism of this activation was found by determining the crystal structure of phosphorylated Aurora A, both with and without a 43-residue domain of TPX2. The data indicated that such domain fully supported kinase activation and protected the T-loop site from dephosphorylation. In the absence of TPX2, the Aurora A activation segment was shown to be in an inactive

conformation, with the crucial phosphothreonine 288 exposed and therefore accessible for dephosphorylation. Mechanistically, binding of TPX2 pulls on the activation segment, swinging the phosphothreonine into a buried position and locking the active conformation, without any global conformational changes in the kinase (Bayliss R. et al, 2003).
Figure 22 presents schematically activation of Aurora A by TPX2.

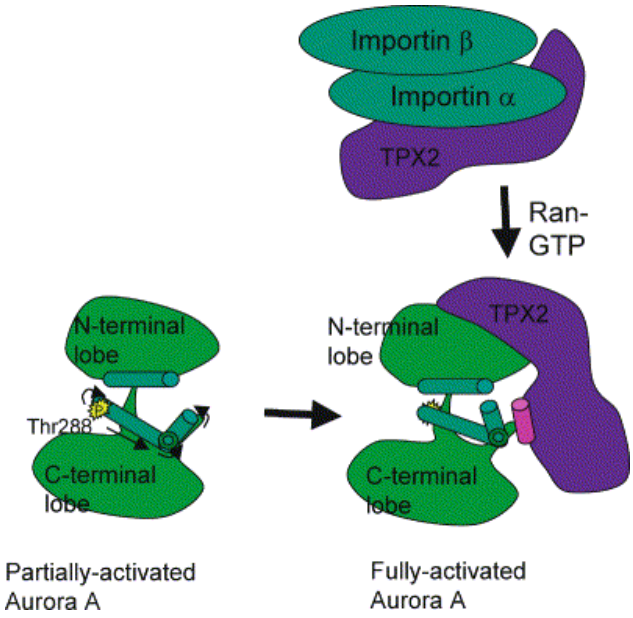


Figure 22. **Activation of Aurora A by TPX2 binding**. The Microtubule-Associated Protein TPX2 Is Released from Its Complex with Importin through the Activity of Ran-GTP. The released molecule makes contact with two regions in the N-terminal lobe of autophosphorylated Aurora A kinase. One of these interactions pulls on a lever arm structure to compact the activation domain and bury the phosphate group on Thr288 and so protect it from hydrolysis by PP1 (after Glover D.M., 2003)

Detailed studies carried out in *Xenopus* revealed the role of G205 as a key site for regulation of TPX2-mediated activation of Aurora A (Bayliss R. et al, 2004). Mutation of this site to N, the equivalent residue in Aurora B, had no effect on autophosphorylation of the T-loop threonine 295 (analog of 288 in human), but instead led to about ten-fold loss of specific activity. Likewise, the opposite mutation in Aurora B (N to G) caused 350-fold increase in activity. G205N Aurora A was found still to be activated by TPX2, but protection of threonine 295 from dephosphorylation by PP1 was abolished. Structural analysis suggested that the presence of G in position 205 results in the movement of the N-terminal domain glycine-rich loop closer to the ATP binding site of the enzyme and also moves the C-helix slightly closer to the activation loop, which would suggest that phosphorylation in the activation loop alone is not sufficient for enzyme activation (Eyers P.A. et al, 2005).

3.2.1.2.2. Inhibition of Aurora A by PP1

Aurora A has two PP1-binding motifs, one that includes the catalytic lysine residue (K^{169}VLF) and a second, immediately adjacent to serine 342 (K^{343}VEF) (Katayama H. et al, 2001). Binding of PP1 to Aurora A is cell cycle regulated and peaks at mitosis. Activated Aurora A phosphorylates PP1 and inhibits its activity *in vitro* as well as *in vivo*, although evidence for direct phosphorylation *in vivo* is lacking. On the other hand, PP1 also was shown to dephosphorylate active Aurora A *in vitro*, and thereby abolish its activity. The postulated mechanism for the Aurora A – PP1 interaction envisages phosphorylation of Aurora A at serine 342 as a mean to decrease binding of PP1 to the K^{343}VEF motif, thus preventing dephosphorylation of threonine 288 and possibly other sites required for Aurora A activity (Littlepage L.E. et al, 2002) (see also Results).
It has also been shown that human PP1 inhibitor 2 (I-2) directly and specifically stimulates recombinant Aurora A activity *in vitro,* in a manner that is independent of PP1inhibition. The C-terminal region of I-2, which is distinct from the primary PP1 binding site, was found to be required for kinase activation, suggesting that two separate regions in I-2 serve two independent functions in Aurora A regulation: one as a PP1 inhibitor and the other as a direct kinase activator (Satinover D.L. et al, 2004).

3.2.1.2.3. Other binding partners influencing Aurora A activity

Screening experiments done in yeast led to identification of another negative regulator of Aurora A, named AIP (Aurora A kinase Interacting Protein) (Kiat L.S. et al, 2002). AIP is a ubiquitously expressed nuclear protein shown to specifically interact with human Aurora A *in vivo*. Ectopic expression of both proteins resulted in the down-regulation of Aurora A protein level and this down-regulation was due to destabilization of the protein through a proteasome-dependent degradation pathway. On the other hand a non-interacting mutant of AIP did not have any effect on Aurora A, showing that the interaction between Aurora A and AIP is important for Aurora A degradation.
A two-hybrid screen identified the LIM protein Ajuba, homolog of a LIM domain-containing protein that promotes meiotic maturation of *Xenopus* oocytes, as an Aurora A binding partner (Hirota T. et al, 2003). Ajuba and Aurora A interact in mitotic cells and become phosphorylated during this interaction. *In vitro* kinase assay with histone H3 as a

substrate showed that Ajuba stimulates Aurora A activity and does so by stimulating autophosphorylation at threonine 288.

Similar type of Aurora A stimulation through binding-induced autophosphorylation - was reported for HEF1, a scaffolding protein, present in spindle asters at mitosis and involved in integrin-dependent attachment signaling at focal adhesions (Pugacheva E.N. and Golemis E.A., 2005).

GSK-3 (glycogen synthase kinase 3) was shown to regulate the activity of *Xenopus* Aurora A (Sarkissian M. et al, 2004). Both proteins were found to interact *in vivo* and GSK-3 was shown to phosphorylate Aurora A at serine 290/291 *in vitro*. This, in turn was shown to promote autophosphorylation of Aurora A at serine 349 (human 342), resulting in a reduction of the kinase activity (see also chapter 3.2.1.1).

The list closes with the only one kinase shown so far to be able to phosphorylate Aurora A *in vivo* – PAK1 (Zhao Z-S. et al, 2005). Active PAK1 was shown to bind to and phosphorylate Aurora A both at threonine 288 and serine 342, promoting the activity of the latter. Thus, in contrast to Ajuba or TPX2, which can only bind to active Aurora A and preserve its active state, PAK1 efficiently binds to inactive Aurora A and induces its activation.

3.2.1.2.4. Inhibition of Aurora A by p53

Using a transactivation-defective mutant as a bait, human p53 protein was shown to directly bind to Aurora A, in a yeast two-hybrid screen (Chen S-S. et al, 2002). The evidence obtained in this study was confirmed *in vivo* by a series of co-immunoprecipitations and GST pull-down assays in which the region of interaction (residues 1 to 318 of p53) was characterized. Interestingly, the N-terminal region of Aurora A containing the Aurora-box, which has been proposed to be a motif for protein-protein interaction (Giet R. and Prigent C., 1999), was sufficient for interaction with p53. The p53-Aurora A interaction resulted in suppression of Aurora A kinase activity *in vitro*. The same transcriptionally inactive p53 can suppress Aurora A-induced centrosome amplification and cellular transformation *in vivo*, further supporting the idea that p53 negatively regulates Aurora A by a direct protein-protein interaction rather than in a transactivation-dependent manner. By contrast, p53 does not suppress transformation induced by Aurora A lacking the N-terminus.

3.2.1.3. Aurora A chemical inhibitors

Aurora kinases are important factors in the control of chromosomal stability and, thus, posses a potential importance in cancer. This thinking was confirmed by the finding that both Aurora A and B are frequently overexpressed in many cancer cells. This has led to the idea that inhibiting the activity of Aurora kinases may have therapeutic utility in cancer. A number of compounds belonging to various structural families have been reported to possess inhibitory activity against Aurora A and to display sufficient selectivity when tested on other protein kinases to allow undertaking both *in vitro* and *in vivo* studies.

ZM447439 (Ditchfield C. et al, 2003), a quinazoline derivative, is an ATP-competitive inhibitor that, in *in vitro* kinase assays, inhibits Aurora A (and B) with IC_{50} values of approximately 0,1 μM. This inhibition is relatively specific, since ZM447439 shows no inhibition (IC_{50} higher then 10 μM) of a range of kinases including Cdk1 and Plk1.

VX-680 (Harrington E.A., et al, 2004), cyclopropane carboxylic acid {4-[4-(4-methyl-piperazin-1-yl)-6-(5-methyl-2H-pyrazol-3-ylamino)-pyrimidin-2-ylsulphanyl]-phenyl}-amide, a small-molecule inhibitor targeting the ATP-binding site, is a potent inhibitor of all three Aurora kinases, with apparent inhibition constant values of 0,6, 18 and 4,6 nM for Aurora A, Aurora B and Aurora C, respectively. VX-680 shows greater than 100-fold selectivity for the Aurora A kinase over 55 other kinases tested, with the only exception for Fms-related tyrosine kinase 3 (FLT-3), the IC50 for which is 30 nM.

JNJ-7706621 (Emanuel S. et al, 2005), a (1,2,4)triazole-3,5-diamine dual Cdk and Aurora inhibitor is able to effectively block cell cycle progression. JNJ-7706621 shows a potent inhibition of several cyclin-dependent kinases and Aurora kinases and selectively blocks proliferation of tumor cells of various origins, independently on p53 and Rb status.

Further chemical modification of known inhibitors (like ZM447439) led to a panel of more selective and potent inhibitors with potential clinical use (Heron N.M, et al, 2006), (Jung F.H., et al, 2006).

3.2.1.4. Degradation

Destruction of Aurora A is dependent on a specialized ubiquitin ligase APC/C-ubiquitin-proteasome pathway (Honda K. et al, 2000). Association of two distinct WD40 repeat proteins, Cdc20 and Cdh1, respectively, sequentially activates APC/C (see chapter 2.3.2). It has been reported that Aurora A degradation is dependent on Cdh1, not on Cdc20 (Taguchi S. et al, 2002), although Cdc20 was found to be associated with Aurora A (Farruggio D.C., 1999). Cdh1, which is also required for the destruction of several mitotic regulatory proteins during late mitosis, binds to two well-characterized APC/C recognition signals: the destruction box and the three-residue KEN sequence. Both signals are highly conserved in Aurora A and Aurora B, although it was shown that Cdh1-dependent degradation of Aurora A does not require the KEN sequence. Determinant for Aurora A degradation are the C-terminal destruction box (Arlot-Bonnemains Y. et al, 2001) and a sequence in the non-catalytic N-terminal domain, the A box (Littlepage L.E. and Ruderman J.V., 2002) (Figure 23).

The A-box is conserved in vertebrate Aurora A, but not in other members of the Aurora family. Serine 53 is phosphorylated during M phase and is part of an A-box. Mutation of this residue to aspartic acid, which can mimic the effect of phosphorylation, completely blocks Cdh1-dependent destruction of Aurora A. This suggests that dephosphorylation of serine 53 during mitotic exit could control the timing of Aurora A destruction by facilitating recognition of both the A-box and the D-box by Cdh1-activated APC/C (Crane R., et al, 2004) (see also chapter 3.2.1.1).

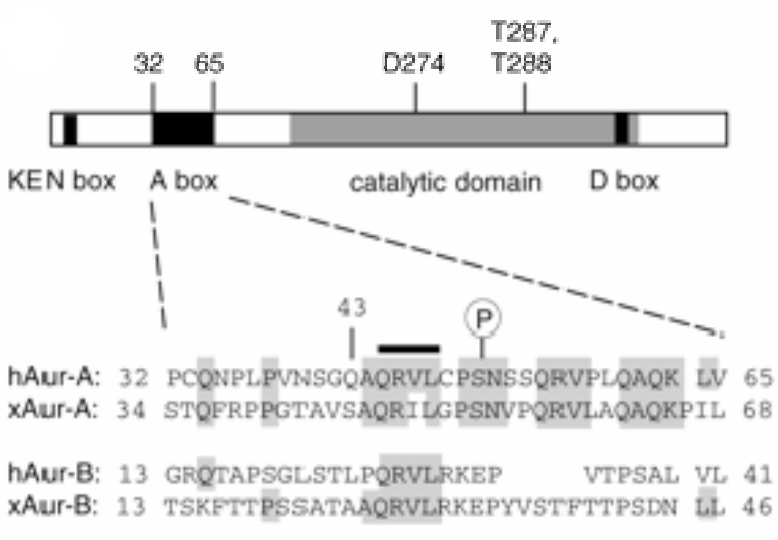


Figure 23. **Schematic of potential destruction signals and catalytic residues in human Aur-A.** The sequence alignment compares the A box in human (h) and *Xenopus* (x) Aur-A with the corresponding region in Aur-B. Residues conserved with human Aur-A are shaded. (after Crane R. et al, 2004)

3.2.2. AurA substrates and targets

In addition to the previously described Aurora A interacting partners (TPX2, PP1, Ajuba), a variety of Aurora A substrates has been identified. I will now describe some of those, that are relevant to Aurora A physiological function and also its potential oncogenic targets.

A two-hybrid screen identified the kinetochore component CENP-A as a protein that interacts with Aurora A (Kunitoku N. et al, 2003). Aurora A was shown to phosphorylate CENP-A *in vitro* on serine 7, a residue that is also phosphorylated by Aurora B. Further analysis showed that Aurora A and Aurora B phosphorylate serine 7 in sequential manner and that Aurora A phosphorylation is required for enrichment of Aurora B at inner centrosomes. Mitotic cells in which this phosphorylation was prevented, exhibited a substantial proportion of misaligned chromosomes as a result of a defect in the ability of kinetochores to attach to microtubules. Phosphorylation of CENP-A by Aurora A in prophase nuclei is thus essential for kinetochore function in mitosis.

TACC3 (Transforming Acidic Coiled Coil 3), a human homologue of the centrosomally associated protein D-TACC, is phosphorylated by Aurora A at serine 558 (Kinoshita K. et al, 2005). TACC3 enhances the number of microtubules emanating from mitotic centrosomes, and its targeting to centrosomes was shown to be regulated by Aurora A-dependent phosphorylation. Another work done in *Drosophila* showed that D-TACC is phosphorylated on serine 863 exclusively at centrosomes during mitosis, and this reaction is carried out by Aurora A (Barros T.P. et al, 2005). Phosphorylation at serine 863 was postulated to play a role in D-TACC-mediated stabilization of microtubules at their minus ends.

Another centrosomal target of Aurora A is Lats2, a serine/threonine kinase, member of Lats kinase family including the *Drosophila* tumor suppressor Lats/Warts. Lats2 was shown to be phosphorylated by Aurora A on serine 83 both *in vitro* and *in vivo* (Toji S. et al, 2004) as well as interact with it and co-localize at centrosomes. The inhibition of Aurora A-induced phosphorylation of Lats2 partially perturbed its centrosomal

localization, suggesting that phosphorylation of Lats2 on serine 83 by Aurora A plays a role in centrosomal localization of Lats2.

It has been shown that BRCA1, the breast cancer tumor suppressor, localizes to the centrosomes and BRCA1 inactivation results in loss of G2/M checkpoint. Aurora A physically binds to BRCA1 in the region 1314-1863 of BRCA1, and phosphorylates it both *in vivo* and *in vitro* at serine 308 (Ouchi M. et al, 2004). Phosphorylation of serine 308 increases in the early M phase, paralleling the increasing level and activity of Aurora A. On the other hand, elimination of Aurora A by siRNA resulted in reduced phosphorylation of BRCA1. Expression of the mutant of this phosphorylation site (S308N) in BRCA1-deficient mouse embryo fibroblasts, decreased the number of cells in M phase to a degree similar to that observed when G2/M arrest was induced by DNA damage in cells expressing wild-type BRCA1, suggesting that phosphorylation of the latter by Aurora A plays a role in the G2/M transition of cell cycle.

The Cdc25B phosphatase, an activator of cyclin-dependent kinase at mitosis, was found to be phosphorylated by Aurora A on serine 353, both *in vitro* and *in vivo* (Dutertre S. et al, 2004). Phosphorylated Cdc25B was found to localize at the centrosomes from prophase to anaphase. This phopshorylation was proposed to participate in the regulation of the entry into mitosis and suggests a local function of Cdc25B as one of the starters of mitosis.

The most proximal known substrate of Aurora A is CPEB, a sequence-specific RNA binding protein, that stimulates cytoplasmic polyadenylation and translational activation (Sarkissian M. et al, 2004). CPEB interacts with the cytoplasmic polyadenylation element (CPE), a *cis*-element present in the 3' untranslated regions (UTRs) of several mRNAs, including those encoding mos and Cyclin B. The translation of mos is necessary for induction the MAP kinase cascade that indirectly activates Cyclin B-Cdk1. Aurora A phosphorylation of CPEB on serine 174 enhances the association of CPEB with CPSF (cleavage and polyadenylation specific factor), possibly helping to stabilize this complex on the AAUAAA hexanucleotide, a second *cis*-element essential for polyadenylation.

HURP (hepatoma upregulating protein) was found as one of the best Aurora A-correlated genes, using the gene expression profiles of Aurora A as a template (Yu C-T.R. et al, 2005). HURP is a cell cycle-regulated gene, highly expressed during G2/M phase, where it

localizes to the spindle fibers, and sharply declines in early to middle G1 phase. Elevated HURP expression is highly associated with HCC, colon and breast cancers, and urinary bladder transitional-cell carcinoma, suggesting a role of HURP in carcinogenesis. Subsequent examination by quantitative RT-PCR confirmed that Aurora A and HURP have similar expression patterns in HCC. Aurora A was shown to phosphorylate HURP in *in vitro* assays, and *in vivo*. This phosphorylation was proposed to modulate HURP function probably by altering its stability or its ability to interact with other proteins.

And last, but not least, p53 has been proven to be a substrate of Aurora A (Katayama H. et al, 2004). Aurora A was shown to phosphorylate p53 at serine 315, which leads to p53 ubiquitination by Mdm2 and eventually its proteolysis. p53 was not degraded in the presence of kinase-inactive Aurora A and silencing of Aurora A resulted in decreased phosphorylation of p53 at serine 315, greater stability of p53 and cell cycle arrest at G2/M. Those results have led to the conclusion that Aurora A kinase is a key regulator of p53 pathway and that its overexpression leads to increased degradation of p53, causing down-regulation of checkpoint-response pathways and facilitating oncogenic transformation of cells. Another phosphorylation site of p53 as target of Aurora A has also been found (Liu Q. et al, 2004). According to the authors, serine 315 in not a major site of Aurora A phosphorylation of p53, instead they found serine 215 to be phosphorylated with higher stoichiometry. This phosphorylation abrogates p53 DNA binding and transactivation activity as was shown by the inhibition of its downstream targets, such as p21 and PTEN. Moreover, the authors have shown that Aurora A-dependent inhibition was exclusively due to its serine's 215 phosphorylation, and not due to phosphorylation of serine 315.

3.2.3 Aurora A in checkpoint control and DNA damage

Cells over-expressing Aurora A were shown to enter anaphase inappropriately, despite defective spindle formation and the persistence of Mad2 at the kinetochores, a fact that creates constant activation of the spindle assembly checkpoint (Anand S. et al, 2003). This effect was reversed by an inhibitory mutant of Bub1, linking the mitotic abnormalities provoked by Aurora A over-expression to spindle checkpoint activity.

The first study linking Aurora A with DNA damage response came out in 2002 (Marumoto T. et al, 2002). The authors showed that Aurora A is inactivated by DNA damage at the end of the G2 phase of the cell cycle and that over-expression of the protein abrogated G2 checkpoint and led to premature entry into mitosis in Rat1 cells. This study had, however, a number of shortcomings that do not allowed the data to be accepted without criticism (see also Results, Chapter 1, Discussion) and that will be dealt with later on in this thesis.
A second study, that was carried out in parallel with ours showed that upon DNA damage Cdc25B is not phosphorylated by Aurora A (see chapter 3.2.2) and Aurora A itself is not activated (Cazales M. et al, 2005). The Authors showed that ectopic expression of Aurora A resulted in bypass of the checkpoint and this was partially overcome by an unphosphorylable Cdc25B mutant, S353A. Finally, the Authors showed that bypass of the G2 checkpoint by the Chk1 kinase inhibitor UCN-01 resulted in the activation of Aurora A kinase and hence phosphorylation of Cdc25B on serine 353.

Since the aim of my study was to elucidate the role of Aurora A in the DNA damage response for further information concerning the subject see Results.

References:

Abraham R.T. "Cell cycle checkpoint signaling through the ATM and ATR kinases", 2001, *Genes and Development*; 15:2177-2196

Abrieu A., Kahana J.A., Wood K.W., Cleveland D.W. "CENP-E as an essential component of the mitotic checkpoint in vitro", 2000, *Cell*; 102:817-826

Adams R.R., Carmena M., Earnshaw W.C. "Chromosomal passengers and the (aurora) ABCs of mitosis", 2001, *Trends in Cell Biology*; 11:49-54

Agalioti T., Lomvardas S., Parekh B., Yie J., Maniatis T., Thanos D. " Ordered recruitment of chromatin modifying and general transcription factors to the IFN-beta promoter", 2000, *Cell*; 103:667-678

Ahn J.Y., Davis H.L., Canman C.E. "Phosphorylation of threonine 68 promotes oligomerization and autophosphorylation of ther Chk2 protein kinase via the forkhead-associated domain", 2002, *The Journal of Biological Chemistry*; 277:19389-19395

Ahonen L.J., Kallio M.J., Daum J.R., Bolton M., Manke I.A., Yaffe M.B., Stukenberg P.T., Gorbsky G.J. "Polo-like kinase 1 creates the tension-sensing 3F3/2 phosphoepitope and modulates the association of spindle-checkpoint proteins at kinetochores", 2005, *Current Opinion in Biology*; 15:1078-1089

Anand S., Penrhyn-Lowe S., Venkitaraman A.R. "Aurora A amplification overrides the mitotic spindle assembly checkpoint, including resistance to Taxol", 2003, *Cancer Cell*; 3:51-62

Ando K., Ozaki T., Yamamoto H., Furuya K., Hosoda M., Hayashi S., Fukuzawa M., Nakagawara A. "Polo-like kinase 1 (Plk1) inhibits p53 by physical interaction and phosphorylation", 2004, *The Journal of Biological Chemistry*; 279:25549-25561

Andrews P.D., Knatko E. Moore W., Swedlow J.R. "Mitotic mechanics: the auroras come into view", 2003, *Current Opinion of Cell Biology*; 15:672-683

Ang X. L., Harper J. W. "SCF-mediated protein degradation and cell cycle control", 2005, *Oncogene*; 24:2860-2870

Antonio C., Ferby I., Wilhelm H., Jones M., Karsenti E., Nebreda A.R., Vernos I. "Xkid, a chromokinesin required for chromosome alignment on the metaphase plate", 2000, *Cell*; 102:425-435

Arlot-Bonnemains Y., Klotzbucher A., Giet R., Uzbekow R., Bihan R., Prigent C. "Identification of a functional destruction box in the Xenopus laevis aurora-A kinase pEg2", 2001, *FEBS Letters*; 508:149-152

Atherton-Fessler S., Parker L.L., Geahlen R.L., Piwnica-Worms H. "Mechanisms of p34cdc2 regulation", 1993, *Molecular and Cellular Biology*; 13:1675-1685

Ayad N.G., Rankin S., Murakami M., Jebenathirajah J., Gygi S., Kirschner M.W. "Tome-1, a trigger of mitotic entry, is degraded during G1 via the APC", 2003, *Cell*; 113:101-113

Bai C., Sen P., Hofmann K., Ma L., Goebl M., Harper W.J., Elledge S.J. "SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box", 1996, *Cell*; 86:263-274

Bakkenist C.J., Kastan M.B. "DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation", 2003, *Nature*; 421:499-506

Barbacid M. "ras genes", 1987, *Annual Rev. Biochemistry*; 56:779-827

Bargonetti J., Manfredi J.J. "Multiple roles of the tumor suppressor p53", 2002, *Current Opinion in Oncology*; 14:86-91

Barros T.P., Kinoshita K., Hyman A.A., Raff J.W. "Aurora A activates D-TACC-Msps complexes exclusively at centrosomes to stabilize centrosomal microtubules", 2005, *The Journal of Cell Biology*; 170:1039-1046

Bartek J., Falck J., Lukas J. "Chk2 kinase – a busy messenger", 2001, *Nature Reviews, Molecular Cell Biology*; 2:877-886

Bartek J., Lukas J. "Chk1 and Chk2 kinases in checkpoint control and cancer", 2003, *Cancer Cell*; 3:421-429

Bartek J., Lukas J. "Mammalian G1- and S-phase checkpoints in response to DNA damage", 2001, *Current Opinion in Cell Biology*; 13:738-747

Bartek J., Lukas C., Lukas J. "Checking on DNA damage in S phase", 2004, *Nature Reviews, Molecular Cell Biology*; 5:792-804

Bayliss R., Sardon T., Ebert J., Lindner D., Vernos I., Conti E. "Determinants for Aurora A and Aurora B discrimination by TPX2", 2004, *Cell Cycle*; 3:404-407

Bayliss R., Sardon T., Vernos I., Conti E. "Structural basis of Aurora A activation by TPX2 at the mitotic spindle", 2003, *Molecular Cell*; 12:851-862

Bell D.W., Varley J.M., Szydlo T.E., Kang D.H., Wahrer D.C.R. Shannon K.E., Lubratovich M., Verselis S.J. Isselbacher K.J., Fraumeni J.F. "Heterozygois germ line hCHK2 mutations in Li-Fraumeni syndrome", 1999, *Science*; 286:2528-2531

Bischoff J.R., Anderson L., Zhu Y., Mossie K., Ng L., Souza B., Schryver B., Flanagan P., Clairvoyant F., Ginther C., Chan C.S.M., Novotny M., Slamon D.J., Plowman G.D. "A homologue of Drosophila aurora kinase is oncogenic and amplified in human colorectal cancers", 1998, *The EMBO Journal*; 17:3052-3065

Bishop J.D., Schumacher J.M. "Phosphorylation of the carboxy terminus of inner centromere protein (INCENP) by the Aurora B kinase stimulates Aurora B kinase activity", 2002, *The Journal of Biological Chemistry*; 31:27577-27580

Blume-Jensen P., Hunter T. "Oncogenic kinase sygnalling", 2001, *Nature*; 411:355-365

Boguski M.S., McCormick F. "Proteins regulating Ras and its relatives", 1993, *Nature*; 366;643-654

Bolano-GarciaV.W. "Molecules in phocus: Aurora kinases", 2005, *The International Journal of Biochemistry and Cell Biology*; 37:1572-1577

Bolton M.A., Lan W., Powers S.E., McCleland M.L., Kuang J., Stukenberg P.T. "Aurora B kinase exists in a complex with survivin and INCENP and its kinase activity is stimulated by survivin binding and phosphorylation", 2002, *Molecular Biology of the Cell*; 13:3064-3077

Brehm A., Miska E.A., Mccance D.J., reid J.L., Bannister A.J., Kouzarides T. "Retinoblastoma protein recruits histone deacetylase to repress transcription", 1998, *Nature*; 391:597-601

Brignone C., Bradley K.E., Kisselev A.F., Grossman S.R. "A post-ubiquitination role of MDM2 and hHR23A in the p53 degradation pathway", 2004, *Oncogene*; 1-9

Brown E.J., Baltimore D. "Essential and dispensable roles of ATR in the cell cycle arrest and genome maintenance", 2003, *Genes and Development*; 17:615-628

Brown J.R., Koretke K.K., Birkeland M.L., Sanseau P., Patrick D.R. “Evolutionary relationship of Aurora kinases; implications for model organisms studies and the development of anti-cancer drugs”, 2004, *BMC Evolutionary Biology*; 4:39-49

Bulavin D.V., Higashimoto Y., Popoff I.J., Gaarde W.A., Basrur V., Potapova O., Appella E., Fornace A.J. "Initiation of a G2/M checkpoint after ultraviolet radiation requires p38 kinase", 2001, *Nature*; 411:102-107

Carmena M., Earnshaw W.C. “The cellular geography of Aurora kinases”, 2003, *Nature*; 4:842-854

Carson C.T., Schwartz R.A., Stracker T.H., Lilley C.E., Lee D.V., Weitzman M.D. "The Mre11 complex is required for ATM activation by DNA damage", 2003, *The EMBO Journal*; 22:6610-6620

Castro A., Arlot-Bonnemains Y., Vigneron S., Labbe J.C., Prigent C., Lorca T. " APC/Fizzy-Related targets Aurora-A kinase for proteolysis", 2002, *EMBO Reports*; 3:457-462

Castro A., Bernis C., Vigneron S., Labbe J-C., Lorca T. "The anaphase-promoting complex: a key factor in the regulation of cell cycle", 2005, *Oncogene*; 24:314-325

Castro A., Vingeron S., Bernis C., Labbe J.C., Lorca T. " Xkid is degraded in a D-box, KEN-box, and A-box-independent pathway", 2003, *Molecular and Cellular Biology*; 23:4126-4138

Cazales M., Schmitt E., Montembault E., Dozier C., Prigent C., Ducommun B. “Cdc25B phosphorylation by Aurora A occurs at the G2/M transition and is inhibited by DNA damage”, 2005, *Cell Cycle*; 4:2-7

Cenciarelli C., Chiaur D.S., Guardavaccaro D., Parks W., Vidal M., Pagano M. " Identification of a family of human F-box proteins", 1999, *Current Opinion in Biology*; 9:1177-1179

Chan G.K., Jablonski S.A., Sudakin V., Hittle J.C., Yen T.J. "Human BUBR1 is a mitotic checkpoint kinase that monitors CENP-E functions at kinetochores and binds the cyclosome/APC", 1999, *Journal of Cell Biology*; 146:941-954

Charles J.F., Jaspersen S.L., Tinker-Kulberg R.L., Hwang L., Szidon A., Morgan D.O. " The Polo-related kinase Cdc5 activates and is destroyed by the mitotic cyclin destruction machinery in S. cerevisiae", 1998, *Current Opinion in Biology*; 8:497-507

Chen J., Jin S., Tahir S.K., Zhang H., Liu X., Sarthy A.V., McGonigal T.P., Liu Z., Rosenberg S.H., Ng S.C. “Survivin enhances Aurora-B kinase activity and localizes Aurora-B in human cells”, 2003, *The Journal of Biological Chemistry*; 278:486-490

Chen S-S., Chang P-C., Cheng Y-W., tang F-M., Lin Y-S. “Suppression of the STK15 oncogenic activity requires a transactivation-independent p53 function”, 2002, *The EMBO Journal*; 21.4491-4499

Cogswell J.P., Brown C.E., Bisi J.E., Neil S.D. “Dominant-negative Polo-like kinase 1 induces mitotic catastrophe independent of cdc25C function”, 2000, *Cell Growth and Differentiation*; 11:615-623

Cortez D., Guntuku S., Qin J., Elledge S.J. "ATR and ATRIP: partners in checkpoint signaling", 2001, *Science*; 294:1713-1716

Costanzo V., Shechter D., Lupardus P.J., Cimprich K.A., Gottesman M., Gautier J. "An ATR- and Cdc7-dependent DNA damage checkpoint that inhibits initiation of DNA replication", 2003, *Molecular Cell*; 11:203-213

Crane R., Gadea B., Littlepage L., Wu H., Ruderman J.V. “Aurora A, meiosis and mitosis”, 2003, *Biology of the Cell*; 96:215-229

Crane R., Kloepfer A., Ruderman J.V. “Requirements for the destruction of human Aurora A”, *Journal of Cell Science*; 117:5975-5983

Crosio C., Fimia G.M., Loury R., Kimura M., Okano Y., Zhou H., Sen S., Allis C.D., Sassone-Corsi P. “Mitotic phosphorylation of histone H3: spatio-temporal regulation by mammalian Aurora kinases”, 2002, *Molecular and Cellular Biology*; 22:874-885

D'Adda di Fagagna F., Reaper P.M., Clay-Farrace L., Fiegler H., Carr P., von Zglinicki T., Saretzki G., Carter N.P., Jaskson S.P. "A DNA damage checkpoint response in telomere-initiated senescence", 2003, *Nature*; 426:194-198

Ditchfield C., Johnson V.L., Tighe A. Ellston R., Haworth C., Johnson T., Mortlock A., Keen N., Taylor S.S. “Aurora B couples chromosome alignment with the anaphase by targeting BubR1, Mad2 and Cenp-E to kinetochores”, 2003, *The Journal of Cell Biology*; 161:267-280

Donzelli M., Draetta G.F. "Regulating mammalian checkpoints through Cdc25 inactivation", 2003, *EMBO Reports*; 4:671-677

Downward J. "Ras signaling and apoptosis", 1998, *Current Oppinion in Genetics and Development*; 8:49-54

Dutertre S., Cazales M., Quaranta M., Froment C., Trabut V., Dozier C., Mirey G., Bouche J-P., Theis-Febvre N., Schmitt E., Monsarrat B., Prigent C., Ducommun B. “Phosphorylation of CDC25B by Aurora A at the centrosome contributes to the G2-M transition”, 2004, *Journal of Cell Science*; 117:2523-2531

Dutertre S., Hamard-Peron E., Cremet J-Y., Thomas Y., Prigent C. "The absence of p53 aggravates polyploidy and centrosome number abnormality induced by Aurora C overexpression", 2005, *Cell Cycle*; 4:136-140

Ellison V., Stillman B. "Opening of the clamp: an intimate view of an ATP driven biological machine", 2001, *Cell*; 106:655-660

Emanuel S., Rugg C.A., Gruninger R.H., Lin R., Fuentes-Pesquera A., Connolly P.J., Wetter S.K., Hollister B., Kruger W.W., Napier C., Jolliffe L., Middletown S.A. “The in vitro and in vivo effects of JNJ-7706621: a dual inhibitor of cyclin-dependent kinases and Aurora kinases”, 2005, *Cancer Research*; 65:90389046

Endicott J.A., Nurse P., Johnson L.N. “Mutational analysis supports a structural model for the cell cycle protein kinase p34”, 1994, *Protein Engineering*; 7:243-253

Evan G.I., Vousden K.H. “Proliferation, cell cycle and apoptosis in cancer”, 2001, *Nature*; 411:342-348

Eyers P.A., Churchill M.E.A., Maller J.L. “The Aurora A and Aurora B protein kinases: a single amino acid difference controls intrinsic activity and activation by TPX2”, 2005, *Cell Cycle*; 4:784789

Eyers P.A., Erikson E., Chen L.G., Maller J.L. “A novel mechanism for activation of the protein kinase Aurora A”, 2003, *Current Biology*, 13:691-697

Eyers P.A., Maller J.L. “Regulation of Xenopus Aurora A activation by TPX2”, 2004, *The Journal of Biological Chemistry*; 279:9008-9015

Falck J., Petrini J.H., Williams B.R., Lukas J., Bartek J. "The DNA damage-dependent intra S-phase checkpoint is regulated by parallel pathways", 2002, *Nature Genetics*; 30:290-294

Fang G. " Checkpoint protein BubR1 acts synergistically with Mad2 to inhibit anaphase-promoting complex", 2002, *Molecular Biology of the Cell*; 13:755-766

Farruggio D.C., Townsley F.M., Ruderman J.V. "Cdc20 associates with the kinase aurora2/Aik", 1999, *PNAS*; 96:7306-7311

Feijoo C., Hall-Jackson C., Wu R., Jenkins D., Leitch J., Gilbert D.M., Smythe C. "Activation of mammalian Chk1 during DNA replication arrest: a role for Chk1 in the intra S-phase checkpoint monitoring replication origin firing", 2001, *Journal of Cell Biology*; 154:913-923

Feldman R.M., Correl C.C., Kaplan K.B., Deshaies R.J. " A complex of Cdc4p, Skp1p, and Cdc53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p", 1997, *Cell*; 91:221-230

Feng Z., Kachnic L., Zhag J., Powell S.N., Xia F. "DNA damage induced p53-dependent BRCA1 nuclear export", 2004, *The Journal of Biological Chemistry*; 279:28574-28584

Ferrari S. "Protein kinase controlling the onset of mitosis", 2006, *Cellular and Molecular Life Science*; 1-15

Ferrari S., Marin O., Pagano M.A., Meggio F., Hess D., El-Shemerly M., Krystyniak A., Pinna L.A. "Aurora A site specificity: a study with synthetic peptide substrates", 2005, *Biochemical Journal*; 390:293-302

Fisher R.P., Morgan D.O. "A novel cyclin associates with MO15/CDK7 to form the CDK-activating kinase", 1994, *Cell*; 78:713-715

Fletcher L., Cerniglia G.J., Nigg E.A, Yend T.J., Muschel R.J. "Inhibition of centrosome separation after DNA damage: a role for Nek2", 2004, *Radiat Research*; 162:128-135

Fry A.M., Mayor T., Meraldi P., Stierhof Y.D., Tanaka K., Nigg E.A. "C-Nap1, a novel centrosomal coiled-coil protein and candidate substrate of the cell cycle-regulated protein kinase Nek2", 1998, *Journal of Cell Biology*; 141:1563-1574

Fry A.M., Schultz S.J., Bartek J., Nigg E.A. "Substrate specificity and cell cycle regulation on Nek2 protein kinase, a potential human homolog of the mitotic regulator NIMA of Aspergillus nidulans", 1995, *Journal of Biological Chemistry*; 270:12899-12905

Gallant P., Nigg E.A. " Cyclin B2 undergoes cell cycle-dependent nuclear translocation and, when expressed as a non-destructible mutant, causes mitotic arrest in HeLa cells", 1992, *Journal of Cell Biology*, 117:213-224

Garret S., Auer K., Compton D.A., Kapoor T.M. "hTPX2 is required for normal spindle morphology and centrosome integrity durng vertebrate cell division", 2002, *Current Biology*; 12:2055-2059

Gatei M., Sloper K., Soerensen C.S., Syljauasen R., Falck J., Hobson J., Zhou B.B., Bartek J., Khanna K.K. "ATM and DBS1 dependent phosphorylation of Chk1 on S317 in response to IR", *Journal of Biological Chemistry*; 278:14806-14811

Geng Y., Eaton E. N., Picon M. "Regulation of cyclin E transcription by E2Fs and retinoblastoma protein", 1996, *Oncogene*; 12:1173-1180

Giet R., Glover D.M. "Drosophila aurora B kinase is required for histone H3 phosphorylation and condensin recruitment during chromosome condensation and to organize the central spindle during cytokinesis.", 2001, *Journal of Cell Biology*; 152:669-82.

Giet R., Petretti C., Prigent C. “Aurora kinases, aneuploidy and cancer, a coincidence or a real link?”, 2005, *Trends in Cell Biology*; 15:241-250

Giet R., Prigent C. “Aurora/Ip1p-related kinases, a new oncogenic family of mitotic serine-threonine kinases”, 1999, *Journal of Cell Science*; 112:3591-3601

Glotzer M. “The molecular requirements for cytokinesis”, 2005, *Science*; 307:1735-1739

Glotzer M., Murray A.W., Kirschner M.W. " Cyclin is degraded by the ubiquitin pathway", 1991, *Nature*; 349:132-138

Glover D.M. “Aurora A on the mitotic spindle is activated by the way it holds its partner”, 2003, *Molecular Cell*, 12:797-799

Glover D.M., Leibowitz M.H., McLean D.A., Parry H. “Mutations in aurora prevent centrosome separation leading to the formation of monopolar spindles”, 1995, *Cell*; 81:95-105

Golan A., Yudkovsky Y., Hershko A. " The cyclin-ubiquitin ligase activity of cyclosome/APC is jointly activated by protein kinases Cdk1-cyclin B and Plk", 2002, *Journal of Biological Chemistry*; 277:15552-15557

Goto H., Kiyono T., Tomono Y., Kawajiri A., Urano T., Furukawa K., Nigg E.A., Inagaki M. “Complex formation of Plk1 and INCENP required for metaphase-anaphase transition”, 2005, *Nature Cell Biology*; 22:1676-1687

Goto H., Yasui Y., Kawajiri A., Nigg E.A., Terada Y., Tatsuka M., Nagata K., Inagaki M. “Aurora-B regulates the cleavage furrow-specific vimentin phosphorylation in the cytokinetic process”, 2003, *The Journal of Biological Chemistry*; 278:8526-8530

Goto H, Yasui Y, Nigg EA, Inagaki M. “Aurora-B phosphorylates Histone H3 at serine28 with regard to the mitotic chromosome condensation”, 2002, *Genes to Cells*; 7:11-17.

Groth A., Lukas J., Nigg E.A., Sillje H.H., Wernstedt C., Bartek J., Hansen K. "Human Tousled like kinase are targeted by an ATM- and Chk1-dependent DNA damage checkpoint”, 2003, *The EMBO Journal*; 22:1676-1687

Gruss O.J., Wittman M., Yokoyama H., Pepperkok R., Kuref T., Sillje H., Karsenti E., Mattaj I.W., Vernos I. “Chromosome-induced microtubule assembly mediated by TPX2 is required for spindle formation in HeLa cells”, 2002, *Nature Cell Biology*; 17:41-49

Hames R.S., Wattam S.L., Yamano H., Bacchieri R., Fry A.M. " APC/C-mediated destruction of the centrosomal kinase Nek2A occurs in early mitosis and depends upon a cyclin A-type D-box", 2001, *EMBO Journal*; 20:7117-7127

Hammond E.M., Denko N.C., Dorie M.J., Abraham R.T., Giaccia A. "Hypoxia links ATR and p53 through replication arrest", 2002, *Molecular Cell Biology*; 22:1834-1843

Harbour J. W., Dean D. C. “The Rb/E2F pathway: expanding roles and emerging paradigms”, 2000, *Genes and Development*; 14:2393-2409

Harbour J.W., Luo R.X., Dei Santi A., Postigo A.A., Dean D.C. " Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1", 1999, *Cell*; 98:859-869

Harrington E.A., Bebbington D., Moore J., Rasnmmussen R.K., Ajose-Adeogun A.O., Nakayama T., Graham J.A., Demur C., Hercend T., Diu-Hercend A., Su M., Golec J.M.C., Miller K.M. “VX-680, a

potent and selective small-molecule inhibitor of the Aurora kinases, suppresses tumor growth in vivo", 2004, *Nature Medicine*; 10:262-266

Hata T., Furukawa T., Sunamura M., Egawa S., Motoi F., Ohmura N., Marumoto T., Saya H., Horii A. "RNA interference targeting Aurora kinase A suppresses tumor growth and enhances the taxane chemosensitivity in human pancreatic camcer cells", 2005, *Cancer Research*; 65:2899-2905

Heffernan T.P., Simpson D.A., Frank A.R., Heinloth A.N., Paules R.S., Codeiro-Stone M., Kaufmann W.K. "An ATR- and Chk1-dependent S checkpoint inhibits replication initiation following UVC-induced DNA damage", 2002, *Molecular Cell Biology*; 22:8552-8561

Hekmat-Nejad M., You Z., Yee M.C., Newport J.W., Cimprich K.A. "Xenopus ATR is a replication-dependent chromatin-binding protein required for the DNA replication checkpoint", 2000, *Current Opinion in Biology*; 10:1565-1573

Helps N.R., Luo X., Braker H.M, Cohen P.T. "NIMA-related kinase 2 (Nek2), a cell-cycle-regulated protein kinase localized to centrosomes, is complexed to protein phosphatase 1", 2000, *Biochemical Journal*; 349:509-518

Heron N.M., Anderson M., Blowers D.P., Breed J., Eden J.M., Green S., Hill G.B., Johnson T., Jung F.H., McMiken H.H.J., Mortlock A.A., Pannifer A.D., Pauptit R.A., Pink J., Roberts N.J., Rowsell S. "SAR and inhibitor complex structure determination of a novel class of potent and specific Aurora kinase inhibitors", 2006, *Bioorganic and Medicinal Chemistry Letters*; 16:1320-1323

Hirota T., Kunitoku N., Sasayama T., Marumoto T., Zhang D., Nitta M., Hatakeyama K., Saya H. "Aurora A an interacting activator, the LIM protein Ajuba, are required for mitotic commitment in human cells", 2003, *Cell*; 114:585-598

Holloway S.L., Glotzer M., King R.W., Murray A.W. " Anaphase is initiated by proteolysis rather than by the inactivation of maturation-promoting factor", 1993, *Cell*; 73:1393-1402

Honda K., Mihara H., Kato Y., Yamaguchi A., Tanaka H., Yasuda H., Furokawa K., Urano T. "Degradation of human Aurora2 protein kinase by the anaphase-promoting complex-ubiquitin-proteasome pathway", 2000, *Oncogene*; 19:2812-2819

Honda R., Korner R., Nigg E.A. "Exploring the functional interactions between Aurora B, INCENP and surviving in mitosis", 2003, *Molecular Biology of the Cell*; 14:3325-3341

Hoyt M.A. "Exit from mitosis: spindle pole power", 2000, *Cell*; 102:267-270

Hsu J.Y., Reimann J.D., Sorensen C.S., Lukas J., Jackson P.K. " E2F-dependent accumulation of hEmi1 regulates S phase entry by inhibiting APC(Cdh1)", 2002, *Nature Cell Biology*; 4:358-366

Hsu JY, Sun ZW, Li X, Reuben M, Tatchell K, Bishop DK, Grushcow JM, Brame CJ, Caldwell JA, Hunt DF, Lin R, Smith MM, Allis CD. "Mitotic phosphorylation of histone H3 is governed by Ipl1/aurora kinase and Glc7/PP1 phosphatase in budding yeast and nematodes.", 2000, *Cell*; 102:279-91.

Huse M., Kuriyan J. "The conformational plasticity of protein kinases", 2002, *Cell*; 109:275-282

Izumi T., Maller J.L. "Elimination of cdc2 phosphorylation sites in the cdc25 phosphatase blocks initiation of M-phase", 1993, *Molecular Biology of the Cell*; 4:1337-1350

Jeggo P.A., Carr A.M., Lehmann A.R. "Splitting the ATM: distinct repair and checkpoint defect in ataxia-teleangiectasia", 1998, *Trends in Genetics*; 14:312-316

Johnson L.N., Lewis R.J. "Structural basis for control by phosphorylation", 2001, *Chemical Reviews*; 101:2209-2242

Johnson S.A., Hunter T. "Kinomics: methods for deciphering the kinome", 2005, *Nature Methods*; 2:17-25

Jung F.H., Pasquet G., Lambert van der Brempt C., Lohmann J-J.M., Warin N., Renaud F., Germain H., Savi C., Roberts N., Johnson T., Dousson C., Hill G.B., Mortlock A.A., Heron N., Wilkinson R.W., Wedge S.R., Heaton S.P., Odedra R., Keen N.J., Green S., Brown E., Thompson K., Brightwell S. "Discovery of novel and potent thiazoloquinazolines as s selective Aurora A and B kinase inhibitors", 2006, *Journal of Medicinal Chemistry*; 49:955-969

Katayama H., Sasai K., Kawai H., Yuan Z-M., Bondarczuk J., Suzuki F., Fujii S., Arlinghaus R.B., Czerniak B., Sen S. "Phosphorylation by aurora kinase A induces Mdm2-mediated destabilization and inhibition of p53", 2004, *Nature Genetics*; 36:55-62

Katayama H., Zhou H., Li Q., Tatsuka M., Sen S. "Interaction and feedback regulation between STK15/BTAK/Aurora A kinase and protein phosphatase 1 through mitotic cell division", 2001, *The Journal of Biological Chemistry*; 276:46219-46224

Kastan M.B., Bartek J. "Cell-cycle checkpoints and cancer", 2004, *Nature*; 432:316-323

Kawajiri A., Yasui Y., Goto H., Tatsuka M., Takahashi M., Nagata K., Inagaki M. "Functional significance of the specific sites phosphorylated in desmin at cleavage furrow: Aurora-B may phosphorylate and regulate type III intermediate filaments during cytokinesis coordinatedly with Rho-kinase", 2003, *Molecular Biology of the Cell*; 14:1489-1500

Kiat L.S., Hui K.M., Gopalan G. "Aurora A kinase interacting protein (AIP), a novel negative regulator of human Aurora A kinase", 2002, *The Journal of Biological Chemistry*; 277:45558-45565

Kim S.T., Xu B., Kastan M.B. "Involvement of the cohesin protein, Smc1, in ATM-dependent and independent response to DNA damage", 2002, *Genes and Development*; 16:571-582

Kimura M., Matsuda Y., Yoshioka T., Okano Y. "Cell cycle dependent expression and centrosome localization of a third human aurora/Ipl1-related protein kinase, AIK3", 1999, *The Journal of Biological Chemistry*; 274:7334-7340

Kinoshita K., Noetzel T.L., Pelletier l., Mechtler K., Drechsel D.N., Schwager A., Lee M., Raff J.W., Hyman A.A. "Aurora A phosphorylation of TACC3/maskin is required for centrosome-dependent microtubule assembly in mitosis", 2005, *The Journal of Cell Biology*; 170:1047-1055

Kitagawa M., Higashi H., Suzuki-Takahashi I. "Phosphorylation of E2F-1 by cyclin A-cdk2", 1995, *Oncogene*; 10:229-236

Kitagawa R., Bakkenist C.J., McKinnon P.J., Kastan M.B. "Phosphorylation of SMC1 is a critical downstream event in the ATM-NBS1-BRCA1 pathway", 2004, *Genes and Development*; 18:1423-1428

Klotzbucher A., Pascreau G., Prigent C., Arlot-Bennemains Y. "A method foe analyzing the ubiquitination and degradation of Aurora A", 2002, *Biological Procedures Online*; 4:62-69

Kotani S., Tugendreich S., Fujii M., Jorgensen P.M., Watanabe N., Hoog C., Hieter P., Todokoro K. " PKA and MPF-activated polo-like kinase regulate anaphase-promoting complex activity and mitosis progression ", 1998, *Molecular Cell*; 1:371-380

Kraft C., Herzog F., Gieffers C., Mechtler K., Hagting A., Pines J., Peters J.M. " Mitotic regulation of the human anaphase-promoting complex by phosphorylation", *The EMBO Journal*; 22:6598-6609

Krause D.R., Jonnalagadda J.C., Gatei M.H., Sillije H.H., Zhou B.B., Nigg E.A., Khanna K. "Suppression of Tousled-like kinase activity after DNA damage or replication block requires ATM, NBS1 and Chk1", 2003, *Oncogene*; 22:5927-5937

Krystyniak A., Garcia-Echeverria C., Prigent C., Ferrari S. "Inhibition of Aurora A in reponse to DNA damage", 2006, *Oncogene*; 1-11

Kufer T.A., Sillje H.W., Körner R., Gruss O.J., Meraldi P., Nigg E.A. "Human TPX2 is required for targeting Aurora A kinase to the spindle", 2002, *The Journal of Cell Biology*; 158:617-623

Kunitoku N., Sasayama T., Marumoto T., Zhang D., Honda S., Kobayashi O., Hatekayama K., Ushio Y., Saya H., Hirota T. "CENP-A phopshorylation by Aurora A in prophase is required foe enrichment of Aurora B at inner centromeres and for kinetochore function", 2003, *Developmental Cell*; 5:853-864

Lammer D., Mathias N., Laplaza J.M., Jiang W., Liu Y., Callis J., Goebl M., Estelle M. " Modification of yeast Cdc53p by the ubiquitin-related protein rub1p affects function of the SCFCdc4 complex", 1998, *Genes and Development*; 12:914-926

Lee C.H., Chung J.H. "The hCds1 (Chk2)-FHA domain is essential for a chain of phosphorylation events on hCds1 that is induced by ionizing radiation", 2001, *Journal of Biological Chemistry*; 276:305376-30541

Lee J.H., Paull T.T. "Direct activation of the ATM protein kinase by the Mre11/Rad50/Nbs1 complex", 2004, *Science*; 304:93-96

Littlepage J.E., Ruderman J.V. "Identification of a new APC/C recognition domain, the A-box, which is required for the Cdh1-dependent destruction of the kinase Aurora A during mitotic exit", 2002, *Genes and Development*; 16:2274-2285

Littlepage J.E., Wu H., Andersson T., Deanehan J.K., Amundadottir L.T., Ruderman J.V. "Identification of phosphorylated residues that affect the activity of the mitotic kinase Aurora A", 2002, *PNAS*; 1-6

Liu F., Stanton J.J., Wu Z., Piwnica-Worms H. "The human Myt1 kinase preferentially phosphorylates cdc2 on threonine 14 and localizes to the endoplasmic reticulum and Golgi complex", 1997, *Molecular and Cellular Biology*; 17:571-583

Liu Q., Kaneko S., Yang L., Feldman R.I., Nicosia S.V., Chen J., Cheng J.Q. "Aurora-A abrogation of p53 DNA binding and transactivation activity by phosphorylation of serine 215", 2004, *The Journal of Biological Chemistry*; 279:52175-52182

Lorca T., Devault A. Colas P., van Loon A., Fesquet D., Lazaro J.B., Doree M. " Cyclin A-Cys41 does not undergo cell cycle-dependent degradation in Xenopus extracts", 1992, *FEBS Letters*; 306:90-93

Lowery D.M., Lim D., Yaffe M.B. "Structure and function of Polo-like kinase", 2005, *Oncogene*; 24:248-259

Luca F.C., Shibuya E.K., Dohrmann C.E., Ruderman J.V. " Both cyclin A delta 60 and B delta 97 are stable and arrest cells in M-phase, but only cyclin B delta 97 turns on cyclin destruction", 1991, *EMBO Journal*; 10:4311-4320

Lukas C., Falck J., Bartkova J., Bartek J., Lukas J. "Distinct spatio-temporal dynamics of mammalian checkpoint regulators induced by DNA damage", 2003, *Nature Cell Biology*; 421:952-960

Lukas J., Lukas C., Bartek J. "Mammalian cell cycle checkpoints: signaling pathways and their organization in space and time", 2004, *DNA Repair 3*, 997-1007

Mailand N. et all "Regulation of G(2)/M events by Cdc25A through phosphorylation-dependent modulation of its stability", 2002, *EMBO Journal*; 21:5911-5920

Mailand N., Falck J., Lukas C., Syljuasen R.G., Welcker M., Bartek J., Lukas J. "Rapid destruction of human Cdc25A in response to DNA damage", 2000, *Science*; 288:1425-1429

Maniatis T. "A ubiquitin ligase complex essential for the NF-κB, Wnt/Wingless and Hedgehog signaling pathways", 1999, *Genes and Development*; 13:505-510

Manning G., Whyte D.G., Martinez R., Hunter T., Sudarsanam S. "The protein kinase complement of the human genome", 2002, *Science*; 298:1912-1934

Marumoto T., Hirota T., Morisaki T., Kunitoku N., Zhang D., Ichikawa Y., Sasayama T., Kuninaka S., Mimori S., Tamaki N., Kimura M., Okano Y., Saya H. "Role of aurora A kinase in mitotic entry and G2 checkpoint in mammalian cells", 2002, *Genes to Cells*; 7:1173-1182

Massague J. "G1 cell-cycle control and cancer", 2004, *Nature*; 432:298-306

Mayo L., Donner D.B., "The PTEN, Mdm2, p53 tumor suppressor – oncoprotein network", 2002, *TRENDS in Biochemical Science*s; 1-6

Melo J., Toczyski A. "A unified view of the DNA-damage checkpoint", 2002, *Current Opinion in Cell Biology*; 14:237-245

Meraldi P., Lukas J., Fry A.M., Bartek J., Nigg E.A. "Centrosome duplication of mammalian somatic cells requires E2F and Cdk2-cyclin A", 1999, *Nature*; 1:88-93

Meraldi P., Nigg E.A. "Centrosome cohesion is regulated by a balance of kinase and phosphatase activities", 2001, *Journal of Cell Science*; 114:3749-3757

Mitra J., Enders G.H., Azizkhan-Clifford J., Lengel K.L. "Dual regulation of the anaphase promoting complex by cyclin A-Cdk2 and cyclin A-Cdk1 complexes", 2006, *Cell Cycle*; 5:6:661-666

Mollinedo F., Gajate C. "Microtubules, microtubule-interfering agents and apoptosis", 2003, *Apoptosis*; 8:413-450

Monaco L., Kolthur-Seetharam U., Loury R., Menissier-de Murcia J., de Murcia G., Sassone-Corsi P. "Inhibition of Aurora B kinase activity by poly(ADP_ribosyl)ation in response to DNA damage", 2005, *PNAS*; 102:14244-14248

Muchardt C., Yaniv M. "When the SWI/SHF complex remodels…the cell cycle", 2001, *Oncogene*; 20:3067-3075

Murata-Hori M., Fumoto K., Fukuta Y., Iwasaki T., Kikuchi A., Tatsuka M., Hosoya H. "Myosin II regulatory light chain as a novel substrate for AIM-1, an aurora/Ipl1p-related kinase from rat", 2000, *The Journal of Biochemistry*; 128:903-907.

Murray A.W., Kirschner M.W. " Cyclin synthesis drives the early embryonic cell cycle", 1989, *Nature*; 339:275-280

Nakanishi K. Taniguchi T., Ranganathan V., New H.V., Moreau L.A., Stotsky M., Mathew C.G., Kastan M.B., Weaver D.T., D'Andrea A.D. "Interactions of FANCD2 and NBS1 in the DNA damage response", 2002, *Nature Cell Biology*; 4:913-920

Nasmyth K., Peters J. M., Uhlmann F. "Splitting the chromosome: cutting the ties that bind sister chromatids", 2000, *Science*; 288:1379-1385

Nigg E.A. "Cyclin-dependent protein kinases: key regulators of the eukaryotic cell cycle", 1995, *BioEssays*; 17:471-480

Nigg E.A. "Mitotic kinase as regulators of the cell division and its checkpoints", 2001, *Nature*; 2:21-32

Nolen B., Taylor S., Ghosh G. "Regulation of protein kinases; controlling activity through activation segment conformation", 2004, *Molecular Cell*; 15:661-675

Norden C., Mendoza M., Dobbelaere J., Kotwaliwale C.V., Biggins S., Barral Y. "The NoCut pathway links completion of cytokinesis to spindle midzone function to prevent chromosome breakage", 2006. *Cell*; 125:85-98

Nyberg K.A., Michelson R.J., Putnam C.W., Weinert T.A. "Toward maintaining the genome: DNA damage and replication checkpoints", 2002, *Annual Reviews in Genetics*; 36:617-656

O'Connell M.J., Walworth N.C., Carr A.M. "The G2-phase DNA damage checkpoint", 2000, *Trends in Cell Biology*; 10:296-303

Ohtsuka T., Jensen M.R., Gu Kim H., Kim K., Lee S.W. "The negative role of cyclin G in ATM-dependent p53 activation", 2004, *Oncogene*; 1-4

Okuda M., Horn H.F., Tarapore P. "Nucleophosmin/B23 is a target of CDK2/cyclinE in centrosome duplication", 2000, *Cell*; 103:127-140

Osborn A.J., Elledge S.J., Zou L. "Checking on the fork: the DNA-replication stress-response pathway", 2002, *Trends in Cell Biology*; 12:509-516

Ouchi M., Fujiuchi N., Sasai K., Katayama H., Minamishima Y.A., Ongusaha P.P., Deng C., Sen S., Lee S.W., Ouchi T. "BRCA1 phosphorylation by Aurora A in the regulation of G2 to M transition", 2004, *The Journal of Biological Chemistry*; 19:19643-19648

Owen D.J., Ornaghi P., Yang J.C., Lowe N., Evans P.R., Ballario P., Neuhaus D., Filetici P., Travers A.A. " The structural basis for the recognition of acetylated histone H4 by the bromodomain of histone acetyltransferase gcn5p", 2000, *EMBO Journal*; 19:6141-6149

Paris J., Philippe M. "Poly(A) metabolism and polysomal recruitment of maternal mRNAs during early Xenopus development", 1990, *Developmental Biology*; 140:221-224

Peeper D.S., Keblusek P., Helin K. "Phosphorylation of a specific cdk site in E2F affect its electrophoretic mobility and promotes pRb-binding in vitro", 1995, *Oncogene*; 10:39-48

Peters J-M. "The anaphase-promoting complex: proteolysis in mitosis and beyond", 2002, *Molecular Cell*; 9:931-943

Peters J.M., King R.W., Hoog C., Kirschner M.W. " Identification of BIME as a subunit of the anaphase-promoting complex", 1996, *Science*; 274:1199-1201

Petrini J.H., Stracker T.H. "The cellular response to DNA double-strand breaks: defining the sensors and mediators", 2003, *Trends in Cell Biology*; 13:458-462

Pinsky B.A., Kung C., Shokat K.M., Biggins S. “The Ipl1-Aurora protein kinase activates the spindle checkpoint by creating unattached kinetochores”, 2006, *Nature Cell Biology*; 8:78-83

Pruitt K., Der C.J. “Ras and Rho regulation of the cell cycle and oncogenesis”, 2001, *Cancer Letters*; 171:1-10

Pugacheva E.N., Golemis E.A. “The focal adhesion scaffolding protein HEF1 regulates activation of the Aurora A and Nek2 kinases at the centrosome”, 2005, *Nature Cell Biology*; 7:937-946

Radhakrishnan S.K., Gartel A.L. “CDK9 phosphorylates p53 on serine residues 33, 315 and 392”, 2006, *Cell Cycle*; 5:519-521

Reed S.I. "Ratchets and clocks: the cell cycle, ubiquitylation and protein turover", 2003, *Nature*; 4:855-864

Reis T., Edgar B.A. “Negative regulation of dE2F1 by cyclin-dependent kinase controls cell cycle timing”, 2004, *Cell*; 117:253-264

Rogers E, Bishop JD, Waddle JA, Schumacher JM, Lin R. “The aurora kinase AIR-2 functions in the release of chromosome cohesion in Caenorhabditis elegans meiosis”, 2002, *The Journal of Cell Biology*; 157:219-229

Royce M.E., Xia W., Sahin A.A., Katayama H., Johnson D.A., Hortobagyi G., Sen S., Hung M-C. “STK15/Aurora A expression in primary breast tumors is correlated with nuclear grade but not with prognosis”, 2003, *Cancer*; 100;12-19

Sanchez I., Dynlacht B.D. “New insights into cyclins, CDKs and cell cycle control”, 2005, *Seminars in Cell and Developmental Biology* 16:311-321

Sarkissian M., Mendez R., Richter J.D. “Progesterone and insulin stimulation of CPEB-dependent polyadenylation is regulated by Aurora A and gycogen synthase kinase-3”, 2004, *Genes and Development*; 18:48-61

Satinover D.L., Leach C.A., Stukenberg T., Brautigan D.L. “Activation of Aurora A kinase by protein phosphatase inhibitor-2, a bifunctional signaling protein”, 2004, *PNAS*; 101:8625-8630

Scian M.J., Stagliano K.E.R., Deb D., Ellis M.A., Carchman E.V., Das A., Valerie K., Deb S.P., Deb S. "Tumor-derived p53 mutants induce oncogenesis by transactivating growth-promoting genes”, 2004, *Oncogene*, 27:4430-4443

Schnitzler G., Sif S., Kingston R.E. " Human SWI/SNF interconverts a nucleosome between its base state and a stable remodeled state", 1998, *Cell*; 94:17-27

Scholey J.M., Brust-Mascher I., Mogilner A. “Cell division”, 2003, *Nature*; 422:746-752

Sens S., Zhou H., White R.A. “A putative serine/threonine kinase encoding gene BTAK on chromosome 20q13 is amplified and overexpressed in human breast cancer cell lines”, 1997, *Oncogene*; 14:2195-2200

Shapiro G.I. “Cyclin-dependent kinase pathways as targets for cancer treatment”, 2006, *Journal of Clinical Oncology*; 24:1770-1783

Sheaff R.J., Groudine M., Gordon M. “Cyclin E-ckd2 is a regulator of $p27^{Kip1}$”, 1997, *Genes and Development*; 11:1464-1478

Shechter D., Constanzo V., Gautier J. "ATR and ATM regulate the timing of the DNA replication origin firing", 2004, *Nature Cell Biology*; 6:648-655

Sherr C.J., Roberts J.M. “CDK inhibitors: positive and negative regulators of G1-phase progression”, 1999, *Genes and Development*; 13:1501-1512

Siloh Y. "ATM and related protein kinases: safeguarding genome integrity", 2003, *Nature Reviews Cancer*; 3:155-168

Siloh Y., Kastan M.B. "ATM: genome stability, neuronal development and cancer cross paths", 2001, *Advanced Cancer Research*; 83:209-254

Sionov R.V., Haupt Y. “The cellular response to p53: the decision between life and death”, 1999, *Oncogene*; 18:6145-6157

Skowyra D., Craig K.L., Tyers M., Elledge S.J., Harper J.W. " F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex", 1997, *Cell*; 91:209-219

Smits V.A.J., Klompmaker R., Arnaud L., Rijksen G., Nigg E.A., Medema R. “Polo-like kinase-1 is a target of the DNA damage checkpoint”, 2000, *Nature Cell Biology*; 2:627-676

Smits V.A.J., Medema R.H. “Checking out the G2/M transition”, 2001, *Biochimica et Biophysica Acta*; 1519:1-12

Song M.S., Song S.J., Ayad N.G., Chang J.S., Lee J.H., Hong H.K., Lee H., Choi N., Kim J., Kim H., Kim J.W., Choi E.J., Kirschner M.W., Lim D.S. " The tumour suppressor RASSF1A regulates mitosis by inhibiting the APC-Cdc20 complex", 2004, *Nature Cell Biology*; 2:129-137

Sorensen C.S., Syljuasen R.G., Falck T., Schroeder L., Ronnstrand K.K., Khanna , Zhou B.B., Bartek J., Lukas J. "Chk1 regulates the S phase checkpoint by coupling the physiological turnover and ionizing radiation-induced accelerated proteolysis of Cdc25A", 2003, *Cancer Cell*; 3:247-258

Staehling-Hampton K., Ciampa P.J., Brook A., Dyson N. " A genetic screen for modifiers of E2F in Drosophila melanogaster", 1999, *Genetics*; 153:275-287

Steward S., Fang G. “Anaphase-promoting complex/cyclosome controls the stability of TPX2 during mitotic exit”, 2005, *Molecular and Cellular Biology*; 10516-10527

Stommel J.M., Wahl G.M. “Accelerated MDM2 auto-degradation induced by DNA-damage kinases is required for p53 activation”, 2004, *The EMBO Journal*; 23:1547-1556

Sugiyama K., Sugiura K., Hara T., Sugimoto K., Shima H., Honda K., Furukawa K., Yamashita S., Urano T. “Aurora B associated protein phosphatases as negative regulators of kinase activation”, 2002, *Oncogene*; 21:3103-3111

Sumara I., Gimenez-Abian J.F., Gerlich D., Hirota T., Kraft C., de la Torre C., Ellenberg J, Peters J.M. “Roles of polo-like kinase 1 in the assembly of functional mitotic spindles”, 2004, *Current Opinion in Biology*; 14:1712-1722

Surana U., Amon A., Dowzer C., McGrew J., Byers B., Nasmyth K. " Destruction of the CDC28/CLB mitotic kinase is not required for the metaphase to anaphase transition in budding yeast", 1993, *EMBO Journal*, 12:1969-1978

Swedlow J.R., Hirano T. “The making of the mitotic chromosome: modern insights into classical questions”, 2003, *Molecular Cell*; 11:557-569

Taguchi S., Honda K., Sugiura K., Yamaguchi A., Furukawa K., Urano T. “Degradation of human Aurora A kinase is mediated by Cdh1”, 2002, *FEBS Letters*; 519:59-65

Taniguchi T., Garcia-Higuera I., Xu B., Andreassen P.R., Gregory R.C., Kim S.T., Lane W.S., Kastan M.B., D'Andrea A.D. "Convergence of the fanconi anaemia and ataxia teleangiectasia signaling pathways", 2002, *Cell*; 109:459-472

Taylor S.S., Ha E., McKeon F. "The human homologue of Bub3 is required for kinetochore localization of Bub1 and a Mad3/Bub1-related protein kinase", 1998, *The Journal of Cell Biology*; 142:1-11

Taylor S.S., McKeon F. "Kinetochore localization of murine Bub1 is required foe normal mitotic timing and checkpoint response to spindle damage", 1997, *Cell*; 89:727-735

Toji S., Yabuta N., Hosomi T., Nishihara S., Kobayashi T., Suzuki S., Tamai K., Nojima H. "The centrosomal protein Lats2 is phosphorylation target of Aurora A kinase", 2004, *Genes to Cells*; 9:383-397

Trieselmann N., Armstrong S., Rauw J., Wilde A. "Ran modulates spindle assembly by regulating a subset of TPX2 and Kid activities including Aurora A activation2, 2003, *Journal of Cell Science*; 116:4791-4798

Tseng T.C., Chen S.H., Hsu Y.P., Tang T.K. "Protein kinase profile of sperm and egg: cloning and characterization of two novel testis-specific protein kinases (AIE1, AIE2) related to yeast and fly chromosome segregation regulators", 1998, *DNA Cell Biology*; 17:823-833

Tseng Y-S., Tzeng C.C., Huang C-Y., Chen P-H., Chiu A. W-H., Hsu P-Y., Huang G-C., Wang Y-C., Liu H-S. "Aurora A overexpression associates with Ha-ras codon-12 mutation and blackfoot disease endemic area in bladder cancer", 2005, *Cancer Letters*, 1-9

Uhlmann F., Wernic D., Poupart M.A., Koonin E.V., Nasmyth K. " Cleavage of cohesin by the CD clan protease separin triggers anaphase in yeast", 2000, *Cell*; 103:375-386

Ulisse S., Delcros J-G., Baldini E., Toller M., Curcio F., Giacomeli L., Prigent C., Ambesi-Impiombato F.S., D'Armiento M., Arlot-Bennemains Y. "Expression of Aurora kinases in human thyroid carcinoma cell lines and tissues", 2006, *International Journal of Cancer*; published on-line 13.02.2006

Uziel T., Lerenthal Y., Moyal L., Andegeko Y., Mittelman L., Shiloh Y. "Requirement of the MRN complex for ATM activation by DNA damage", 2003, *EMBO Journal*; 22:5612-5621

van Vugt M.A.T.M., Bras A., Medema R.H. "Polo-like kinase-1 controls recovery from a G2 damage-induced arrest in mammalian cells", 2004, *Molecular Cell*; 15:799-811

van Vugt M.A.T.M., Bras A., Medema R.H. "Restarting the cell cycle when the chceckpoint comes to a halt", 2005, *Cancer Research*; 65(16):7037-7040

Vousden K.H, Lu X. "Live or let die: the cell's response to p53", 2002, *Nature*; 2:594-604

Wakefield J.G., Stephens D.J., Tavare J.M. "A role for glycogen synthase kinase-3 in mitotic spindle dynamics and chromosome alignment", 2002, *Journal of Cell Science*; 116:637-646

Walter A.O., Seghezzi W., Korver W., Sheung J., Lees E. "The mitotic serine/threonine kinase Aurora 2/AIK is regulated by phosphorylation and degradation", 2000, *Oncogene*; 19:4906-4916

Walworth N.C. "Cell-cycle checkpoint kinases: checking in on the cell cycle", 2000, *Current Opinion in Cell Biology*, 12:697-704

Watanabe N., Arai H., Nishihara Y., Taniguchi M., Watanabe N., Hunter T., Osada H. "M-phase kinases induce phospho-dependent ubiquitination of somatic Wee1 by $SCF^{\beta\text{-}TrCP}$", 2004, *PNAS*; 101:4419-4424

Winston J.T., Strack P., Beer-Romero P., Chu C.Y., Elledge S.J., Harper J.W. " The SCFbeta-TRCP-ubiquitin ligase complex associates specifically with phosphorylated destruction motifs in IkappaBalpha and beta-catenin and stimulates IkappaBalpha ubiquitination in vitro", 1999, *Genes and Development*; 13:270-283

Wu G., Xu G., Schulman B.A., Jeffrey P.D., Harper J.W., Pavletich N.P. "Structure of a β-TRCP1-Skp1-β-Catenin complex: destruction motif binding and lysine specificity of the $SCF^{\beta\text{-}TrCP1}$ ubiquitin ligase", 2003, *Molecular Cell*; 11:1445-1456

Xiao Z., Chen Z., Gunasekera A.H., Sowin T.J., Rosengerg S.H., Fesik S., Zhang H. "Chk1 mediates S and G2 arrest through Cdc25A degradation in response to DNA-damaging agents", 2003, *Journal of Biological Chemistry*; 278:21767-21773

Xu X., Tsvetkov L., Stern D. "Chk2 activation and phosphorylation-dependent oligomerization", 2002, *Molecular and Cellular Biology*; 22:4419-4432

Yakushijin Y., Hamada M., Yasukawa M. "The expression of the Aurora A gene and its significance with tumorigenesis in Non-Hodgkin's lymphoma", 2004, *Leukemia and Lymphoma*; 45:1741-1746

Yamaguchi T., Goto H., Yokoyama T., Sillje H., Hanish A., Uldschmid A., takai Y., Oguri T., Nigg E.A., Inagaki M. "Phosphorylation by Cdk1 induces Plk1-mediated vimetin phosphorylation during mitosis", 2005, *The Journal of Cell Biology*; 171:431-436

Yamasaki L., Pagano M. "Cell cycle, proteolysis and cancer", 2004, *Current Opinion in Cell Biology*; 16:623-628

Yan X., Cao L., Li Q., Wu Y., Zhang H., Saiyin H., Liu X., Zhang X., Shi Q., Yu L. "Aurora C is directly associated with Survivin and required for cytokinesis", 2005, *Genes to Cells*; 10:617-626

Yang H., Ou C.C., Feldman R.I., Nicosia S.V., Kruk P.A., Cheng J.Q. "Aurora A kinase regulates telomerase activity through c-Myc in human ovarian and breast epithelial cells", 2004, *Cancer Research*; 64:463-467

Yao X., Abrieu A., Zheng Y., Sullivan K.F., Cleveland D.W. "CENP-E forms a link between attachment of spindle microtubules to kinetochores and the mitotic checkpoint", 2000, *Nature Cell Biology*; 2:484-491

Yarden R.I., Pardo-Reoyo S., Sgagias M., Cowan K.H., Brody L.C. "BRCA1 regulates the G2/M checkpoint by activationg Chk1 kinase upon DNA damage", 2002, *Nature Genetics*; 30:285-289

Yu C-T., Hsu J-M., Lee Y-C.G., Tsou A-P., Chou C-K., Huang C-Y.F. "Phosphorylation and stabilization of HURP by Aurora A: implication of HURP as a transforming target of Aurora A", 2005, *Molecular and Cellular Biology*; 25:5789-5800

Yuan J-H., feng Y., Fisher R., Maloid S., Longo D.L., Ferris D.K. "Polo-like kinase 1 inactivation following mitotic DNA damaging treatments is independent of Ataxia Teleangiectasia Mutated kinase", 2004, Molecular *Cancer Research*; 2:417-426

Zachariae W., Shevchenko A., Andrews P.D., Ciosk R., Galova M., Stark M.J., Mann M., Nasmyth K. " Mass spectrometric analysis of the anaphase-promoting complex from yeast: identification of a subunit related to cullins", 1998, *Science*; 279:1216-1219

Zhao H., Watkins H., Piwnica-Worms H. "Disruption of the checkpoint kinase 1/cell division cycle 25A pathway abrogates ionizing radiation-induced S and G2 checkpoints", 2002, *PNAS*; 99:14795-14800

Zhao J., Dynlacht B., Imai T. "Expression of NPAT, a novel substrate of cyclin E-cdk2, promotes S-phase entry", 1998, *Genes and Development*; 12:2298-2313

Zhao Z-S., Lim J.P., Ng Y-W., Lim L., Manser E. "The GIT-associated kinase PAK targets to the centrosome and regulates Aurora A", 2005, *Molecular Cell*; 20:237-249

Zheng N., Wang P., Jeffrey P.D., Pavletich N.P. " Structure of a c-Cbl-UbcH7 complex: RING domain function in ubiquitin-protein ligases", 2000, *Cell*; 102:533-539

Zhou B.B., Elledge S.J. "The DNA damage response: putting checkpoints in perspective", 2000, *Nature*; 408:433-439

Zou L., Elledge S.J. "Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes", 2003, *Science*; 300:1542-1548

AIM OF STUDY

The faithful replication and segregation of chromosomes as part of the cell division cycle requires that topological changes be imposed upon cellular DNA. Interconversion of different topological forms of DNA is catalyzed by DNA topoisomerases, a family of enzymes classified according to their catalytic mechanism of action. Type I enzymes introduce transient single-stranded breaks into DNA, pass an intact single strand of DNA through the broken strand, and then re-ligate the break. Type II enzymes, in contrast, make transient double-stranded breaks into one segment of DNA and pass an intact duplex through the broken DNA, before resealing the break (reviewed by Watt P.M. and Hickson I.D., 1994).

Topoisomerase II is required for the viability of all eukaryotic cells and plays important roles in DNA replication, recombination, transcription, chromosome segregation and the maintenance of the nuclear scaffold. In human and other mammalian cells, there are at least two forms (α and ß) of the topoisomerase II enzyme (Tsai-Pflugfelder, M. et al, 1988) (Jenkins, J.R. at al, 1992). Topoisomerase II catalyses a transient double-stranded break in the DNA helix, allowing the passing of a second double strand of DNA through the break, which is then re-ligated. Topoisomerase poisons acts by prolonging the lifetime of these open intermediate 'cleavable complexes' forming obstructions that eventually lead to DNA damage (Fortune, J.M. and Osheroff, N., 2000). DNA damage upon double-strand breakage is normally sensed by ATM or ATR kinase complexes. These kinases directly phosphorylate the checkpoint kinases Chk1 and Chk2 that, in turn, phosphorylate Cdc25 family members causing their inactivation by nuclear exclusion or degradation. The DNA damage signal mediated via Chk1 and Chk2 also regulates Cyclin B/Cdk1, Wee1 and other proteins involved in the G2/M transition, changing their expression, phosphorylation and subcellular localization (see Introduction).

Topoisomerase II is a specific target for several clinically important anti-tumour drugs such as anthracyclines, e.g. adriamycin and daunorubicin, epipodophyllotoxins, e.g. etoposide and teniposide, anthracenedione, e.g. mitoxantrone, and aminoacridines, e.g. m-AMSA (reviewed by Andoh T. and Ishida R., 1998). These compounds exert cytotoxic effects by stabilizing covalent complexes between topoisomerase II and DNA, the so-

called 'cleavable complex', thus generating DNA double-strand breaks. They are often referred to as topoisomerase II poisons, because they convert the enzyme to DNA-cleaving toxins in conjunction with the agents. The cell killing mechanism of these 'classical' topoisomerase II inhibitors is believed to be related to enzyme-mediated DNA cleavage, and the mechanism(s) by which DNA damage leads to cell death is still under intense investigation (see (Binaschi M. et al, 1995) and (Watt P.M. and Hickson I.D., 1994) for reviews).

It has been recently discovered that kinases involved in proper spindle formation play important roles in the cellular response to DNA damage. The role of Nek-2 and Plk1 in both DNA damage checkpoint and cell cycle arrest has already been elucidated (as described in Introduction).

Much less is known about the involvement of Aurora A in DNA damage response. Published data is incomplete and reports are conflicting – for example in case of the issue of the relationship between Aurora A and Cyclin B/Cdk1 one study placed Aurora A as dependent on Cyclin B/Cdk1 (Marumoto T. et al, 2002) whereas an other proposed an opposite view, claiming that Aurora A was required for promoting Cyclin B/Cdk1 activation (Hirota T. et al, 2003). The latter scenario seemed to us more likely since Aurora A overexpression correlates with tumour formation and checkpoint abrogation (see Introduction).

The lack of clear picture on the mechanism controlling Aurora A prompted us to investigate the role of Aurora A as possible effector in the cellular response to DNA damage.

In my study I used etoposide (Figure 1) as an agent creating double-strand breaks in DNA.

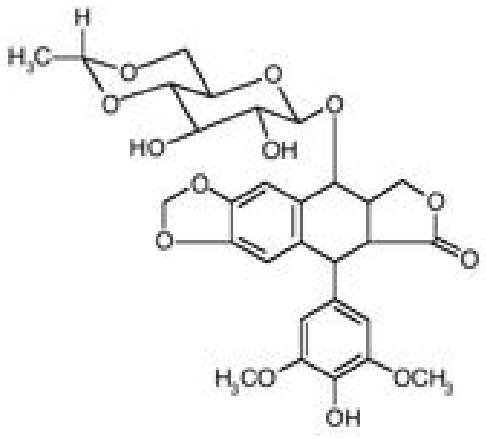


Figure 1. **Etoposide**
4'-demethyl-epipodophyllotoxin, 4,6-O-ethylidene-beta-D- glucopyranoside (8CI)
(picture from Wikipedia
http://en.wikipedia.org/wiki/Etoposide)

The reason for choosing etoposide, rather than ionizing radiation, was essentially technical (i.e. simplicity of administration and possibility to treat several sets of culture plates at once). On the other hand, I was able to get comparable results using either etoposide or IR, which allowed me to conclude that the response of Aurora A is specific to the presence of double-strand breaks, rather than being a stress response to the etoposide treatment.

References:

Binaschi M., Zunino F., Capranico G., "Mechanism of action of DNA topoisomerase inhibitors", 1995, *Stem Cells*, **13**, 369-379.

Fortune, J.M. and Osheroff, N. "Topoisomerase II as a target for anticancer drugs: when enzymes stop being nice", 2000, *Prog. Nucleic Acid Res. Mol. Biol*, **64**, 221–253

Hirota T., Kunitoku N., Sasayama T., Marumoto T., Zhang D., Nitta M., Hatakeyama K., Saya H. "Aurora A an interacting activator, the LIM protein Ajuba, are required for mitotic commitment in human cells", 2003, *Cell*; 114:585-598

Jenkins, J.R., Ayton, P., Jones, T., Davies, S.L., Simmons, D.L., Harris, A.L., Sheer, D., Hickson, I.D. "Isolation of cDNA clones encoding the beta isozyme of human DNA topoisomerase II and localization of the gene to chromosome 3p24", 1992, *Nucleic Acids Res*, **20**, 5587–5592

Marumoto T., Hirota T., Morisaki T., Kunitoku N., Zhang D., Ichikawa Y., Sasayama T., Kuninaka S., Mimori S., Tamaki N., Kimura M., Okano Y., Saya H. "Role of aurora A kinase in mitotic entry and G2 checkpoint in mammalian cells", 2002*, Genes to Cells*; 7:1173-1182

Smits V.A.J., Klompmaker R., Arnaud L., Rijksen G., Nigg E.A., Medema R. "Polo-like kinase-1 is a target of the DNA damage checkpoint", 2000, *Nature Cell Biology*; 2:627-676

Tsai-Pflugfelder, M., Liu, L.F., Liu, A.A., Tewey, K.M., Whang-Peng, J., Knutsen, T., Huebner, K., Croce, C.M., Wang, J.C. "Cloning and sequencing of cDNA encoding human DNA topoisomerase II and localization of the gene to chromosome region 17q21-22", 1988, *Proc. Natl Acad. Sci. USA*, **85**, 7177–7181

Watt P.M., Hickson D.I. "Structure and function of type II DNA topoisomerases", 1994, *Biochem. J.* **303**, 681-695

Wikipedia, http://en.wikipedia.org/wiki/Etoposide

RESULTS

1. INHIBITION OF AURORA A IN RESPONSE TO DNA DAMAGE

AGNIESZKA KRYSTYNIAK, C. GARCIA-ECHEVERRIA, C. PRIGENT and S. FERRARI

Reprinted from: *Oncogene, 2006, Jan 19; 25 (3):338-48*

Oncogene (2005), 1–11

www.nature.com/onc

ORIGINAL ARTICLE

Inhibition of Aurora A in response to DNA damage

A Krystyniak[1], C Garcia-Echeverria[2], C Prigent[3] and S Ferrari[1]

[1]*Institute of Molecular Cancer Research, University of Zurich, Zurich, Switzerland;* [2]*Department of Oncology, Novartis Pharmaceuticals, Basel, Switzerland and* [3]*CNRS UMR6061, Cell Cycle Group, University of Rennes 1, Rennes Cedex, France*

Mitotic kinases are the ultimate target of pathways sensing genotoxic damage and impinging on the cell cycle machinery. Here, we provide evidence that Aurora A (AurA) was inhibited upon generation of double-strand breaks in DNA. We demonstrate that AurA was not downstream of CDK1 and that inhibition of AurA and CDK1 by DNA damage occurred independently. Using a cell line functionally deficient in Chk2, a selective Chk1 inhibitor and siRNA to Chk1, we show that DNA-damage signals were delivered to AurA through a Chk1-dependent pathway. With regard to the molecular mechanism of AurA inhibition, we found that the point mutation Ser_{342} > Ala rendered AurA resistant to inhibition by DNA damage. By means of two distinct approaches we examined the impact of reconstitution of AurA activity in DNA-damaged cells: (i) transient expression of wild-type and Ser_{342} > Ala mutant, but not kinase-dead, AurA led to bypass of the DNA damage block; (ii) direct transduction of highly active wt-AurA into G2 arrested cells precisely after induction of DNA damage resulted in mitotic entry. We show that the mechanism through which AurA allowed entry into mitosis was reactivation of CDK1, thus indicating that AurA plays a key role upstream of CDK1. A model depicting the possible role of AurA at the onset of mitosis and upon DNA damage is presented.
Oncogene advance online publication, 12 September 2005; doi:10.1038/sj.onc.1209056

Keywords: AurA; CDK1; DNA damage; mitosis; phosphorylation

Introduction

Genome stability is prerequisite to faithful transmission of genetic information to the progeny. Errors in transmission of heritable information derive from problems intrinsic to the machinery dedicated to DNA synthesis and DNA repair, or originate from incorrect segregation of chromatids during mitosis (Lengauer *et al.*, 1998; Loeb *et al.*, 2003). In normal cells, spontaneous and induced DNA damage is sensed by a network of proteins that direct the assembly of DNA repair protein complexes and generate signals delaying the onset of mitosis, a process known as 'DNA damage response' (Nyberg *et al.*, 2002). Organisms as distant as yeast and mammals display a remarkably similar DNA damage response, indicating that the mechanisms sensing and addressing correction of DNA damage have been conserved throughout evolution. In budding yeast, the central regulators of the pathway are Mec1p and Tel1p, two members of the PI-3K subfamily of protein kinases. Orthologs of Mec1p and Tel1p in humans are the protein kinases ATR and ATM. The former responds to many types of damage and is believed to be of key importance in the surveillance of DNA replication, while the latter appears to be primarily involved in the response to double-strand breaks (DSB) (Rouse and Jackson, 2002). Upon detection of damage by specific protein complexes, ATR/ATM are recruited to the damaged sites, where they facilitate assembly of repair factors concomitantly with the activation of two downstream protein kinases, Chk1 and Chk2. These kinases contribute on the one hand to the phosphorylation of repair factors, and on the other hand target and inactivate Cdc25C and CDK1, two key components of the cell cycle machinery. As compared to yeast, additional controls such as stabilization of p53, nuclear exclusion of cyclin B and inhibition of the mitosis-promoting Polo-like kinase add further complexity to the cell cycle arrest triggered in response to DNA damage in mammals. Aurora proteins were first described in yeast and *Drosophila* and belong to a novel subfamily of mitotic protein kinases that regulate centrosomal and microtubule activity, thus controlling the accuracy of chromosome segregation and cytokinesis (Giet and Prigent, 1999). One single form of the kinase, Ipl1, is expressed in budding yeast (Chan and Botstein, 1993), where mutants display abnormal ploidy (Biggins *et al.*, 1999). *Drosophila* Aurora mutants typically display monopolar spindles as a result of defective centrosome separation (Glover *et al.*, 1995). A number of Aurora homologues have been identified in other organisms (Giet and Prigent, 1999). Of the three human homologues so far described, Aurora A (AurA) and Aurora B (AurB) were isolated during the screening for kinases overexpressed in colon carcinoma (Bischoff *et al.*, 1998). AurA kinase activity

Correspondence: Dr S Ferrari, Institute of Molecular Cancer Research, University of Zurich, Winterthurerstrasse 190, Zurich CH-8057, Switzerland.
E-mail: sferrari@imcr.unizh.ch
Received 4 May 2005; revised 15 July 2005; accepted 19 July 2005

peaks at G2, before the activation of AurB (Bischoff *et al.*, 1998). AurA is localized to the centrosomes during interphase and to the spindle throughout mitosis. Analysis of AurA function in HeLa cells by siRNA revealed that depletion of AurA results in an almost complete block of entry into mitosis (Hirota *et al.*, 2003). Overexpression of AurA was shown to cause transformation of Rat1 and NIH3T3 cells, which in turn could grow as tumors in nude mice (Bischoff *et al.*, 1998). Biochemically, full activation of AurA requires phosphorylation at a number of sites by as yet unknown upstream kinases (Walter *et al.*, 2000; Littlepage *et al.*, 2002) as well as autophosphorylation (Haydon *et al.*, 2003; Ferrari *et al.*, 2005). AurA interacts with protein phosphatase 1 (PP1), which appears to contribute to regulation of AurA kinase activity during mitosis (Katayama *et al.*, 2001). Inactivation of AurA by ubiquitin-dependent degradation occurs at mitotic exit and is mediated by the anaphase-promoting complex/cyclosome (APC/C) (Honda *et al.*, 2000; Castro *et al.*, 2002; Littlepage and Ruderman, 2002). Recent studies on the potential role of AurA in cancerogenesis have described the frequent amplification of AurA gene in human tumors and cancer cell lines as well as the high expression of AurA mRNA in a manner independent of gene amplification (Sen *et al.*, 1997; Bischoff *et al.*, 1998; Zhou *et al.*, 1998).

Considering that DNA damage triggers arrest before cell division and that AurA plays a key role at the onset of mitosis, we set out to investigate whether regulation of AurA is a target of DNA damage-triggered pathways. We have addressed the eventual dependence of AurA from CDK1 and then examined the pathway relaying DNA damage signals to AurA by means of specific kinase inhibitors, cells functionally deficient in checkpoint kinases and siRNA. Finally, in ectopic expression as well as protein transduction experiments, we addressed the question on the ability of active AurA to promote bypass of the G2 block imposed by DNA damage. We show that this occurred through AurA-driven CDK1 reactivation and suggest a model that integrates these novel findings into the network of mitotic signaling pathways.

Materials and methods

Expression vectors, chemicals, peptides and antibodies

Full-length AurA was obtained by means of PCR using Pfu-polymerase (Stratagene). The first-strand cDNA template was synthesized on polyA$^+$ mRNA purified from mitotic HeLa cells using M-MLV reverse transcriptase (Promega). The gene-specific forward and reverse primers used in the PCR reactions were 5′-CGCGGATCCATGGACCGATCTAAAGAAAACT GCATTTC-3′ and 5′-GGCGAGCTCCTAAGACTGTTTGC TAGCTGATTCTTTG-3′, respectively. The 1.2 Kb PCR product was subcloned into pBluescript SK$^+$ (Stratagene) via *BamHI*/*Xho*I sites and controlled by sequencing. Recombinant AurA was generated by subcloning AurA ORF in pTXB3 (New England BioLabs) followed by expression of the Intein–AurA fusion protein in the BL21 *Escherichia coli* strain and one-step affinity purification on Chitin beads. A polyclonal serum to AurA (AurA-Pab36) was generated in two rabbits (Clonestar, Brno, Czech Republic) using full-length AurA. IgGs were purified by FPLC on a Protein A-Sepharose column (Amersham-Pharmacia). The purified polyclonal antibody to AurB (#3094) was from Cell Signaling Technology.

Expression vectors for myc-tagged forms of wild-type (wt) and kinase-dead (D145 > N) AurA in pCS2 were kindly provided by P Sassone-Corsi (Strasbourg, France). The S342 > A mutant was generated using the QuickChange site-directed mutagenesis kit (Stratagene). Monoclonal antibody 9E10 to the myc-tag was from Santa Cruz Biotechnology. UCN-01 was dissolved in DMSO. The kinase substrate myelin basic protein (MBP) was purchased at Sigma.

The carrier peptide Pep-1 (Morris *et al.*, 2001) was synthesized on a Milligen 9050 Plus automated peptide synthesizer (continuous flow) using chemical protocols based on Fmoc chemistry. The purity of the final compound was verified by reversed-phase analytical HPLC and the identity was assessed by correct mass spectral and amino acid analyses.

Cell culture

HeLa, HEK-293T and U2-OS were maintained in DMEM (OmniLab) supplemented with 10% fetal calf serum (FCS, Life Technologies), penicillin (100 U/ml) and streptomycin (100 μg/ml). HCT-15 cells were maintained in RPMI-1640 containing 20% FCS and antibiotics. For synchronization experiments cells were seeded at 1×10^6 in 10 cm plates and treated after 24 h with 2 mM thymidine (SynGen Inc.) for 16 h, released for 8 h and thymidine (2 mM) was added for a second period (15 h). When indicated cells were synchronized by addition of hydroxyurea (2 mM) for 15 h. The extent of synchronization was controlled by flow cytometric analysis of DNA. Transient transfections were performed using FuGene6 (Roche).

Immunofluorescence

Indirect immunofluorescence experiments were performed with cells grown on acid-washed glass cover slips as described (Charrasse *et al.*, 2000). Detection of AurA, centrin or histone H3 pospho-Ser$_{10}$ were performed with purified AurA-Pab36 antibody (1/800), monoclonal antibody to centrin (1/2000) and polyclonal antibody to H3-pS$_{10}$ (1/100). FITC-labeled (1/750) or TRITC-labeled (1/50) secondary antibodies were combined with DAPI (Molecular Probes). Cells were observed with a Leica DMRB microscope equipped with a 100 W HBO lamp for fluorescence. High-resolution pictures were taken with oil-immersion lenses (PL-FLUOTAR 40 × –100 ×) and images were captured with a Leica DC 200 camera. Cells were viewed using Leica DC Viewer software and image merging was obtained using Adobe Photoshop 7.0.

Western blotting, immunoprecipitation and protein kinase assay

Cell extraction and detection of proteins by Western blot analysis were carried out as previously described (Charrasse *et al.*, 2000). Immunoprecipitations were carried out for 3 h at 4°C in Buffer A (50 mM Tris–HCl, pH 7.5, 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 6 mM EGTA, 15 mM Na-pyrophosphate, 0.5 mM Na-orthovanadate, 1 mM benzamidine, 0.1 mM phenylmethylsulfonil fluoride (PMSF), 1% Nonidet P-40) using 100, 200 or 500 μg of total protein for CDK1, Chk2 or AurA, respectively. Proteins were immobilized on Protein A/G-Agarose beads (S Cruz Biotech.) and washed in 3 × 1 ml ice-cold Buffer A. AurA was routinely immunoprecipitated with purified AurA-Pab36 antibody and deteced by Western blot with monoclonal antibody 35C1. To assess CDK1 or AurA protein kinase activity, immunoprecipitates were additionally

washed with 1 × 1 ml Buffer B (20 mM Tris-HCl, pH 7.5, 0.5 M LiCl) and with 1 × 1 ml Buffer C (20 mM Tris–HCl, pH 7.5, 1 mM dithiothreitol (DTT), 100 mM NaCl, 10 mM $MgCl_2$). Pellets were dried and resuspended in a final volume of 20 μl Buffer C containing the kinase substrate (0.1 mg/ml) (MBP, myelin basic protein) and 50 μM [γ-^{32}P]ATP (Amersham-Pharmacia) (specific activity: 5–10 μCi/nmol for AurA and 2 μCi/nmol for CDK1 kinase assay, respectively). Autophosphorylation of AurA was performed as indicated above with the exception that MBP was omitted. In the case of Chk2, immunoprecipitates were washed in 3 × 1 ml ice-cold Buffer A followed by 1 × 1 ml Buffer D (10 mM MOPS pH 7.0, 0.2 mM EDTA, 10 mM $MgCl_2$). Pellets were dried and resuspended in a final volume of 20 μl Buffer D containing the kinase substrate (200 μM Cdc25 peptide, as described in Davies *et al*. (2000)) and 100 μM [γ-^{32}P]ATP (Amersham-Pharmacia) (specific activity 1.0 μCi/nmol).

siRNA treatment

HeLa cells were seeded in triplicate wells of a six-well plate and treated with siRNA to Chk1 (Ambion validated siRNA #51 147) for 48 h. Synchronization by double thymidine was performed during treatment with siRNA. Chk1 expression was detected with a rabbit polyclonal antibody (Cell Signaling Technology #2345) or the monoclonal antibody DSC-310 (a kind gift of A Kraemer and J Lukas).

Results

Effect of genotoxic agents on AurA

Given the function of AurA at the onset of mitosis we asked whether it might be a target of DNA damage signals. AurA protein level was low in interphase and raised during progression to mitosis (Figure 1a), as previously reported (Bischoff *et al*., 1998; Stenoien *et al*., 2003). Using purified AurA-Pab36 antibody, which did not show crossreactivity with AurB (Supplemental data Figure 1), we confirmed that AurA was present at centrosomes during S and G2 phases and at spindle poles during mitosis (Supplemental data Figure 1). In order to examine the impact of DNA damage on AurA we employed etoposide (Supplemental data Figure 2; Nghiem *et al*., 2001), which causes DSB in DNA by inhibiting topoisomerase II, thus impairing chromosome condensation and decatenation of sister chromatids (Andoh and Ishida, 1998). To avoid eventual unspecific effects of the drug on AurA synthesis we administered etoposide to double-thymidine synchronized HeLa cells after completion of DNA synthesis (8 h postrelease from the second thymidine block), when AurA level was close to maximum. The treatment caused arrest at G2, as shown by flow cytometry (Figure 1b, top panel) and lack of histone H3 phosphorylation (Figure 1b, bottom panel and Supplemental data Figure 3). Such a block was characterized by high levels of cyclins A and B1 as well as inhibition of CDK1 activity (Figure 1c). Under these conditions, AurA accumulated to high levels (Figure 1c), in a manner similar to the two cyclins. We excluded any possible toxicity of etoposide, since long treatment with the drug did not result in apoptosis, as judged by the lack of the sub-G1 peak in the flow

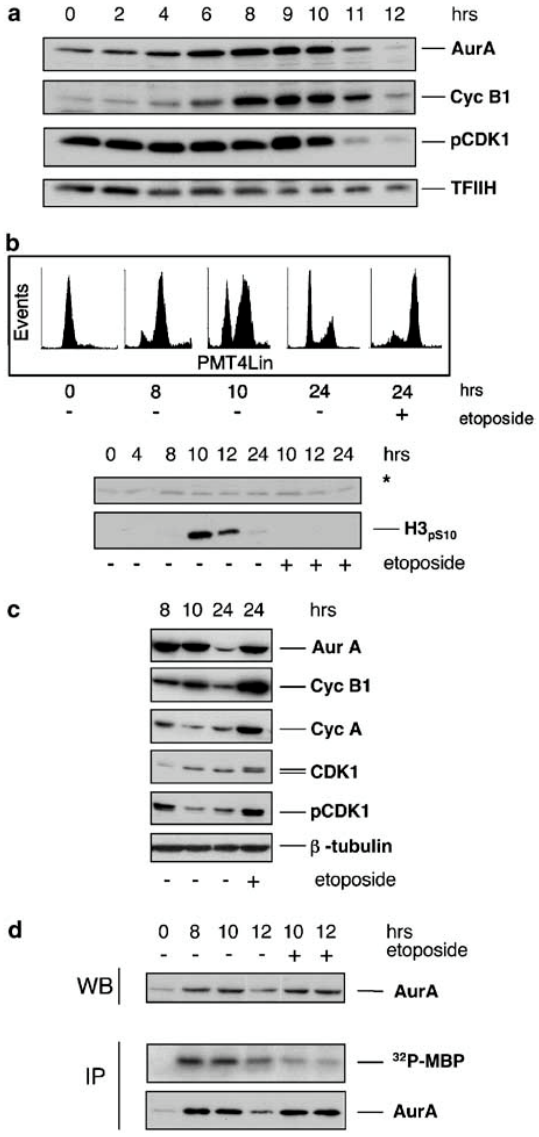


Figure 1 Damage to DNA affects AurA turnover and kinase activity. (**a**) Detection of AurA, Cyclin B1 and phosphorylated CDK1 by Western blot analysis of whole-cell extracts (WCE) derived from double-thymidine synchronized HeLa cells. TFIIH was used as loading control. (**b**) Synchronized HeLa cells were treated in the presence or the absence of etoposide (5 μM) at 8 h postrelease and analysed by flow cytometry at the indicated time (top panel). Histone H3 phosphorylation was used as read-out for entry into mitosis (bottom panel). The asterisk denotes an unspecific band taken as loading control. (**c**) Expression of AurA and cell cycle markers were examined in synchronized cells treated in the presence or the absence of etoposide as described in B. (**d**) Determination of AurA kinase activity upon treatment of cells with etoposide. Upper panel: AurA protein as detected in WCE using purified rabbit AurA-Pab36 antibody; middle panel: AurA was immunoprecipitated with purified rabbit AurA-Pab36 antibody and subjectd to protein kinase activity assay; lower panel: immunoprecipitated AurA was detected with monoclonal antibody 35C1.

cytometry profile (Figure 1b). Addition of etoposide at 10 h postrelease, that is when sister chromatids are decatenated and cells transit through mitosis (Supplemental data Figure 1, middle panels), did not result in accumulation of AurA and cyclin B1 and, accordingly, did not block transition through mitosis (data not shown). This indicated that the effect of etoposide was related to the generation of DSB in DNA during the process of decatenation of sister chromatids and that the accumulation of AurA was not merely the result of unspecific stress responses in the cell (see also below). We next asked whether DNA damage affected AurA kinase activity. Immunoprecipitation of AurA from etoposide-treated cells showed that kinase activity was inhibited (Figure 1d). Visual inspection of etoposide-treated cells confirmed that cells were impaired in progression to mitosis (Figure 2). The magnification displayed in Figure 2 shows that etoposide treatment did not block duplication, but rather separation of the centrosomes, a process contributed to by AurA (Nigg, 2001).

AurA is activated independently of CDK1

It is well established that activation of cyclin B1/CDK1 plays a key role during mitotic entry. Since CDK1 inactivation is held responsible for the G2/M block resulting from DNA damage (Smits and Medema, 2001), we asked whether the accumulation of inactive AurA that we observed in response to damage was related to CDK1 inhibition. As prerequisite to this study, we first examined whether AurA and CDK1 activities were linked during the regular progression to mitosis, since in the literature there is conflicting evidence on this issue (Bischoff *et al*., 1998; Marumoto *et al*., 2002; Hirota *et al*., 2003) (see 'Discussion'). To this end, we employed roscovitine, a di-methyl derivative of olomoucine that displays the same mechanism of action of olomoucine but is more selective (De Azevedo *et al*., 1997). Treatment of synchronized cells with roscovitine led to accumulation of cells at G2 (Figure 3C, right panel and Figure 3D). AurA protein did not undergo degradation in roscovitine-arrested cells, as opposed to untreated cells (Figure 3A, top panel lane 3 vs lane 2) that were regularly transiting through mitosis (Figure 3C, left panel). This was expected, considering that inhibition of CDK1 is known to block turnover of mitotic proteins by the APC/C. Immunoprecipitation of AurA (Figure 3B, top panel) or CDK1 (Figure 3B, middle panel) followed by determination of protein kinase activity showed that roscovitine treatment led to effective inhibition of CDK1, but left AurA activity unchanged. Accordingly, visual inspection of roscovitine-treated cells reflected the fact that AurA accumulation at centrosomes proceeded undisturbed, centrosomes separated normally (Figure 3D) and the centrosome structure was not disrupted (Figure 3E). Under these conditions, however, cells displayed no mitotic figures, in agreement with the fact that CDK1 activity was inhibited (Kimura *et al*., 1998). Lack of dependency of AurA activity from CDK1 was further inferred by analysis of Tyr_{15} phosphorylation in CDK1, which clearly dropped after the observed peak of AurA protein (Figure 1a) and activity (Figure 1d). Taken together these data demonstrated that, in somatic cells, AurA activity is independent of CDK1. These data further imply that etoposide-mediated inhibition of AurA in G2 likely occurred through a mechanism independent of CDK1.

Signaling damage to AurA is mediated through Chk1

The next issue that we addressed was to clarify whether the effect of etoposide on AurA was mediated through transducers of DNA damage. To this end, we employed the colon cancer cell line HCT-15. These cells are heterogeneous with regard to Chk2, which is either not expressed or is mutated and does not respond to DNA damage (Falck *et al*., 2001). In fact, we observed that Chk2 electrophoretic mobility and kinase activity were

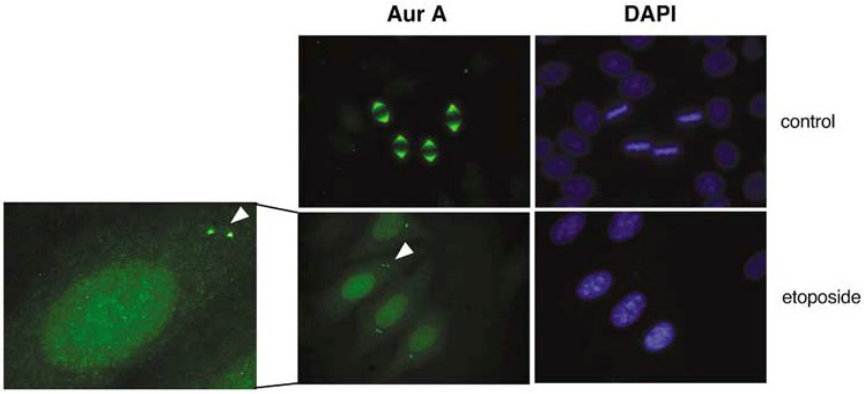


Figure 2 Effect of DNA damage on AurA localization and centrosome separation. HeLa cells synchronized by double-thymidine block were treated with etoposide (5 μM) at 8 h postrelease and analysed for expression and localization of AurA with purified rabbit AurA-Pab36 antibody at 10 h. Nuclei were visualized by DAPI staining of DNA. White arrows show duplicated centrosomes.

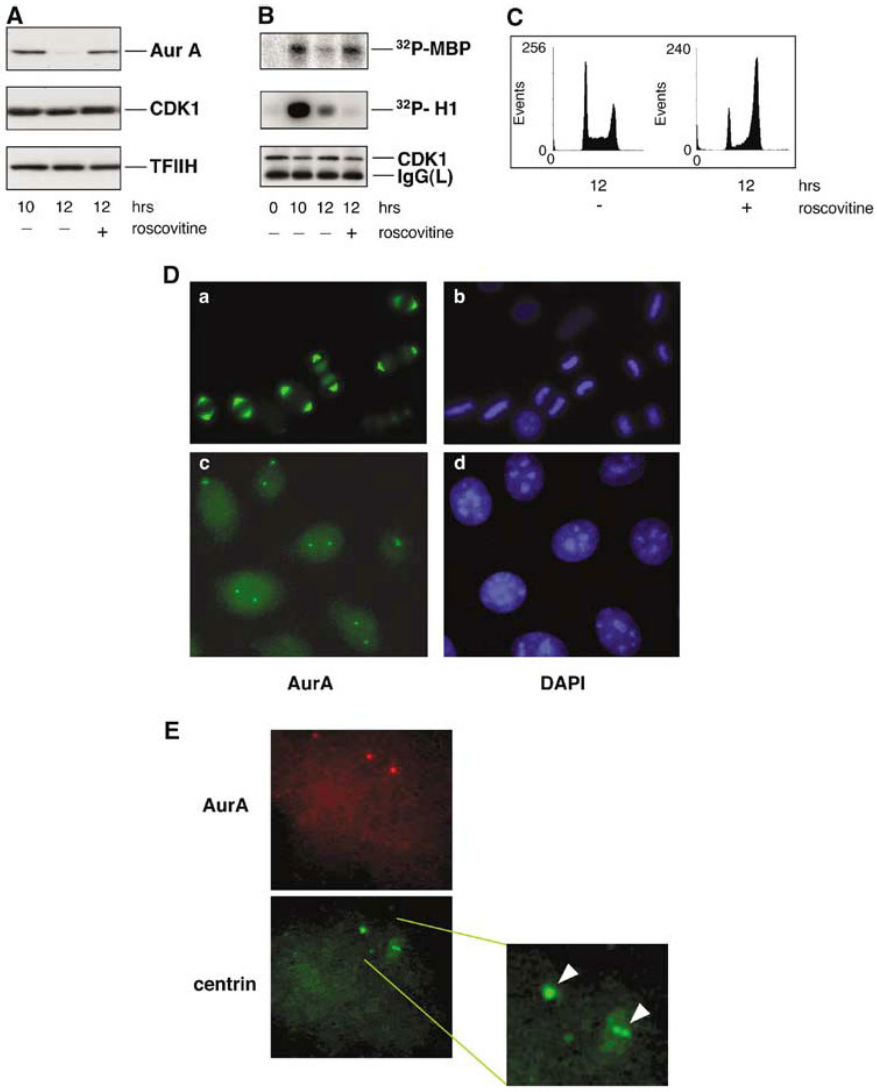
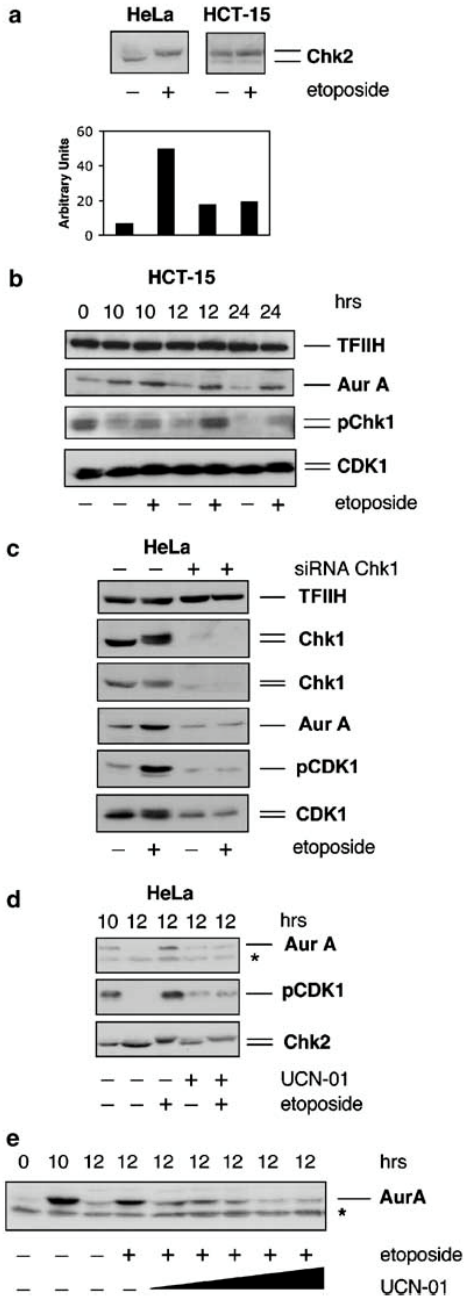


Figure 3 AurA activity and mitotic function are independent of CDK1. HeLa cells synchronized by double-thymidine block release were treated with roscovitine (40 μM) at 6 h. (**A**) AurA and CDK1 expression at the indicated time were determined by Western blot. (**B**) Protein kinase activity of imunoprecipitated AurA and CDK1 was determined as described under Materials and methods. Western blot analysis of immunoprecipitated CDK1 is shown as control for the immunoprecipitation (**B**, bottom panel). The effect of roscovitine on cell cycle progression (**C**) and on AurA localization (**D**) was assessed by flow cytometric analysis and immunofluorescence, respectively. AurA and DAPI staining in untreated (a and b) or roscovitine-treated cells (c and d) are shown. (**E**) Staining of centrin was used to document the presence of two centrioles at centrosomes.

not affected by etoposide in HCT-15 cells, contrary to HeLa cells that express functional Chk2 (Figure 4a). Double-thymidine synchronized HCT-15 cells (Figure 4b) displayed the same pattern of cell cycle- and DNA damage-dependent AurA accumulation observed in HeLa cells. Considering that in HCT-15 cells Chk1 was activated by DNA damage (Figure 4b), signaling to AurA and CDK1 in these cells was likely mediated through Chk1. To further substantiate this finding we employed siRNA to Chk1 or the Chk1 inhibitor UCN-01 (Zhao *et al*., 2002). Reduced expression of Chk1 by siRNA as well as inhibition of Chk1 kinase activity by UCN-01 prior to the generation of DNA damage completely abolished the accumulation of AurA and tyrosine-phosphorylated CDK1 (Figure 4c and d). A slight reduction of CDK1 upon treatment of cells with siRNA to Chk1 was observed using two independent oligonucleotides. Off-target effects were, however, ruled out by the specific sequence of the siRNA chosen and by the detection of a number of other proteins like Chk2, SHC-A, SHC-B, SHC-C (data not shown) and TFIIH (Figure 4c). The effect of UCN-01 could be appreciated

at doses as low as 5 nM (Figure 4e, lane 5 vs lane 4), indicating the specificity of the inhibition. The fact that Chk2 mobility shift was unaffected by treatment of cells with UCN-01 (Figure 4d, lane 5) confirmed the lack of Chk2 involvement in the pathway controlling AurA. Immunofluorescence data confirmed that in cells treated with UCN-01 progression to mitosis occurred despite damage to DNA (Supplemental data Figure 4).



Active AurA overrides DNA damage signals

The evidence presented above indicated that AurA, like CDK1, is a downstream target of DNA damage-triggered pathways. Considering the important role of AurA at the onset of mitosis, we asked whether the presence of active AurA in etoposide-blocked cells might be sufficient to bypass the block. To this end, we considered possible mutations that may render AurA resistant to inhibitory signals. A study on *Xenopus laevis* AurA identified three *in vitro* sites of phosphorylation with roles in protein stability and activity (Littlepage *et al*., 2002). In particular, mutation of Ser_{349} (equivalent to Ser_{342} in human AurA) to Asp was shown to result in an inactive kinase, whereas the Ala_{349} isoform displayed activity similar to wt-AurA. This indicated that phosphorylation at Ser_{349} in *X. laevis* AurA might be inhibitory (Littlepage *et al*., 2002). We constructed a nonphosphorylatable human AurA mutant ($S_{342}>A$) and transiently expressed it in HEK 293T cells. As observed for *X. laevis* AurA, the human $S_{342}>A$ displayed activity similar to the wt-kinase (Figure 5a, lane 3 vs 1). Assessment of AurA activity in cells ectopically expressing wt-, kd- or A_{342}-AurA isoforms and treated with etoposide revealed that A_{342}-AurA remained active (Figure 5b, lane 6) whereas wt-AurA was partially inhibited (Figure 5b, lane 3). Incomplete inhibition of transiently expressed wt-AurA by DNA damage when compared with endogenous AurA (Figure 1d, lanes 5 and 6) could be explained considering that overexpression of exogenous AurA likely saturated the inhibitory signals triggered by etoposide. The cell cycle distribution of synchronized HEK 293T cells ectopically expressing AurA isoforms was unaffected in the absence of DNA damage (data not shown). In DNA-damaged cells, however, the ectopically expressed wt- and kd-AurA displayed an opposite behavior: whereas wt-AurA facilitated bypass of the

Figure 4 DNA damage signal to AurA is relayed through Chk1. (**a**) Exponentially growing HeLa or HCT-15 cells were treated with etoposide (5 μM) for 4 h and Chk2 electrophoretic mobility (top) or kinase activity (bottom) was examined. (**b**) HCT-15 cells synchronized by double-thymidine block were treated in the presence of etoposide (5 μM) at 8 h postrelease and analysed at the indicated time points. The high level of Chk1-S_{345} phosphorylation at time 0 h likely reflected intra S phase checkpoint activation by stalled replication forks. (**c**) HeLa cells were treated with control or Chk1 siRNA and double-thymidine synchronized. Etoposide (5 μM) was given at 8 h and proteins analysed by Western blotting at 24 h postrelease. Expression of Chk1 was monitored using a rabbit polyclonal (top) or the DSC-310 mouse monoclonal antibody (bottom). (**d**) Synchronized HeLa cells were treated with the Chk1 inhibitor UCN-01 (300 nM) at 7.5 h postrelease. Etoposide (5 μM) was added at 8 h and cells were analysed at the indicated time points. Phosphorylation of CDK1 at Tyr_{15} was used as biochemical marker for the effect of UCN-01 (middle panel). The asterisk in AurA blot indicates a nonspecifically reacting protein that was taken as loading control. (**e**) Double-thymidine synchronized HeLa cells were treated with increasing amounts of UCN-01 (5 10 50 100 300 nM, lanes 5 9) at time 7.5 h postrelease and DNA damage was generated by addition of etoposide (5 μM) at 8 h. The expression of AurA was examined at the indicated time points. The asterisk in AurA blot indicates a nonspecifically reacting protein that was taken as loading control.

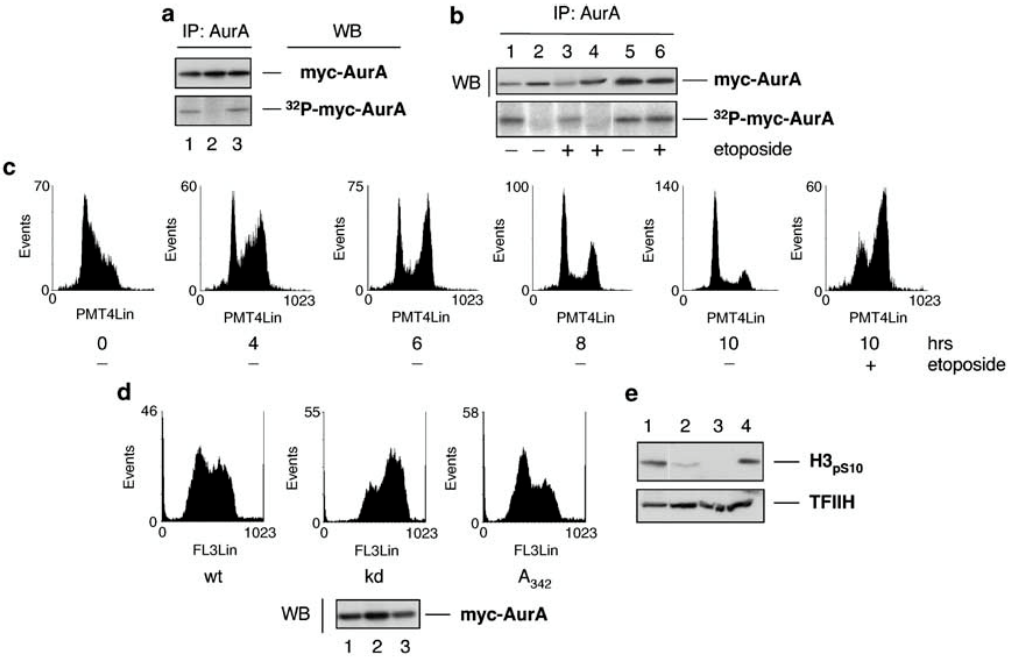
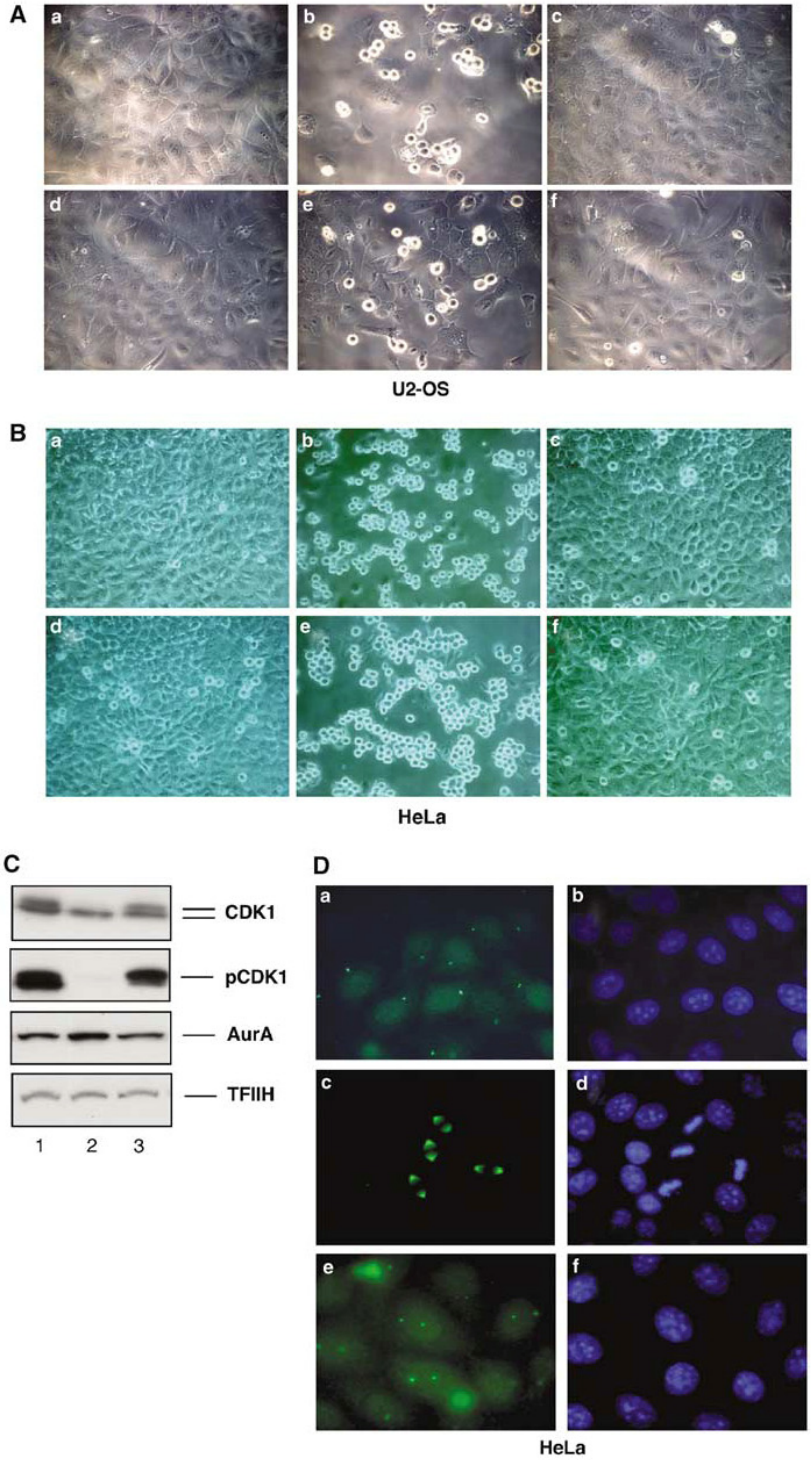


Figure 5 Ectopic expression of wild-type (wt) AurA can bypass the G2 block caused by DNA damage. (**a**) Immunoprecipitation of myc-tagged forms of wt-AurA (lane 1), kd-AurA (lane 2) and A_{342}-AurA (lane 3) with monoclonal antibody 9E10 from extracts of transiently transfected HEK 293T cells. Protein expression was revealed with purified AurA-Pab36 antibody (upper panel) and kinase activity (lower panel) was determined as described in Materials and methods. (**b**) Myc-tagged wt- (lanes 1 and 3), kd- (lanes 2 and 4) or A_{342}-AurA (lanes 5 and 6) were immunoprecipitated from synchronized 293T cells using monoclonal antibody 9E10. Protein was detected with purified rabbit AurA-Pab36 antibody (upper panels) and kinase activity (lower panels) was assayed as described in Materials and methods. (**c**) Flow cytometric analysis of double-thymidine block-released HEK 293T cells. (**d**) Synchronized HEK 293T cells were transfected with myc-tagged wt-AurA, kd-AurA or A_{342}-AurA (as indicated) between the two thymidine blocks. DNA damage was generated by addition of etoposide (5 μM) 3 h following release from the second block, and cells were analysed by flow cytometry at 10 h (upper panels). Expression of myc-AurA in cells that were analysed by flow cytometric analysis is shown (lower panel, lane 1: wt-AurA; lane 2: kd-AurA; lane 3: A_{342}-AurA). (**e**) Synchronized HEK 293T cells were treated with nocodazole to prevent progression beyond M phase (lane 1). Cells transfected with wt-AurA (lane 2), kd-AurA (lane 3) or A_{342}-AurA (lane 4) as shown in (**d**) were treated with nocodazole and etoposide (5 μM) 3 h following release from the second block and analysed 10 h postrelease. The ability of AurA isoforms to promote entry into mitosis was scored by detection of phosphorylated Ser_{10} in histone H3.

etoposide-induced G2 block, as evidenced by an increase in the G1 population, kd-AurA was unable to promote transition through mitosis (Figure 5d, upper panels). A_{342}-AurA resulted to be more efficient than the wt kinase in facilitating progression through mitosis (Figure 5d, upper panels). Detection of phosphorylation at Ser_{10} in histone H3 documented mitotic entry of DNA-damaged cells transfected with wt- or A_{342}-AurA (Figure 5e). Given the drawback of large overexpression of proteins in transient transfection assays, we decided to use a system that allows rapid transduction of proteins in the cell in a manner compatible with the kinetic of G2 arrest by DNA damage signals. To this end, we transduced human recombinant wt- and kd-AurA into HCT-116, HeLa and U2-OS cells. Recombinant AurA isoforms were purified to homogeneity upon expression in *E. coli*. The wt-kinase displayed high specific activity whereas the kinase-dead form was completely inactive (Ferrari *et al*., 2005). Synchronized U2-OS (Figure 6A) or HeLa cells (Figure 6B) were treated either with etoposide, nocodazole or both compounds, and the onset of mitosis was scored by the number of rounded-up cells at 20 h postrelease. Whereas etoposide impaired entry into mitosis, nocodazole-treated cells appeared to be mitotic (U2-OS $\approx 45 \pm 5\%$; HeLa $\approx 90 \pm 5\%$) at the time point examined. Transduction of etoposide-treated cells with active AurA at 9 h postrelease could rescue cells from the G2 block and advance them into mitosis (U2-OS $\approx 17 \pm 6.1\%$; HeLa $\approx 84 \pm 8\%$). Quantification of the band corresponding to AurA (Supplemental data Figure 5) showed that the exogenous protein transduced into cells was a fraction ($\sim 40\%$) of the physiological amount of endogenous AurA. To document progression to mitosis, we examined staining of transduced wt-AurA to the poles of the mitotic spindle in HeLa (Figure 6D) and HCT-116 cells (data not shown). Omission of nocodazole in this set of experiments resulted in an apparently lower number of cells bypassing the block at the time point examined. Nonetheless, a similar number of mitotic cells could be detected at subsequent time points (data not shown), an indication of the different rate of mitotic entry in a population of cells. In all cases we observed that transduction of active AurA caused

nuclear membrane breakdown, correct positioning of centrosomes and formation of the mitotic spindle despite the presence of DNA damage. Analysis of CDK1 phosphorylation at Tyr_{15} showed that DNA damage-dependent inhibition of CDK1 was relieved in cells transduced with active AurA (Figure 6C). Finally, treatment of wt-AurA-transduced cells with roscovitine (Figure 6B, panel f and 6d, panels e,f) abolished entry



into mitosis, pointing to the need of active CDK1 in this process. Taken together, these results showed that catalytically active AurA, but not the kinase-dead mutant, triggered CDK1 reactivation and allowed overriding the DNA damage checkpoint.

Discussion

Mitosis is an exquisitely regulated process resulting in the segregation of sister chromatids into two newly formed cells (Nigg, 2001). Although initial evidence indicated that CDK1 is the master regulator of mitosis, subsequent studies have shown the role of Polo-like (Plk1), NIMA- and Aurora-related protein kinases in the commitment to and execution of mitosis (Giet and Prigent, 1999; Nigg, 2001; Hirota *et al*., 2003; Marumoto *et al*., 2003). Considering that DNA damage results in the block of mitosis, we set out to investigate whether inactivation of AurA is a part of the cellular response to genotoxic damage. Our data indicate that DNA damage causing arrest at the G2 transition of the cell division cycle resulted in accumulation of AurA protein, likely as a result of APC/Cyclosome malfunction (Peters, 2002). The unlikelihood of transcriptional induction of AurA in this context is supported by the finding that transcription of genes encoding mitotic regulators, including AurA, is rapidly turned off following DNA damage (Crawford and Piwnica-Worms, 2001). We observed that AurA accumulated in DNA-damaged cells was devoid of kinase activity. Synchronization of cells at G2 with roscovitine did not result in inhibition of AurA kinase activity, thus ruling out the possibility that AurA inhibition by DNA damage could have been merely the consequence of cell cycle synchronization. Visual inspection of etoposide-treated cells confirmed the lack of centrosome separation and mitotic figures.

In order to establish whether DNA damage-dependent AurA inhibition may have been the consequence of CDK1 inhibition, the next issue that we addressed was the link between AurA and CDK1. Initial studies established that activation of AurA precedes the peak of CDK1 activity (Bischoff *et al*., 1998). A later report, however, claimed that activation of AurA depends on CDK1 (Marumoto *et al*., 2002), although the conclusion drawn in this study was made using chemical inhibitors of CDK1 at concentrations that may have had unpredictable side-effects *in vivo* (i.e. on the pathway of AurA synthesis). As support to this, titration studies that we performed with the more selective CDK1 inhibitor roscovitine showed that a high concentration of the compound ($>100\,\mu$M) led to a dramatic reduction of AurA protein (data not shown). Further studies on AurA and CDK1 regulation reached the conclusion that AurA is required for promoting cyclin B1/CDK1 activation (Hirota *et al*., 2003). Controversial conclusions on this issue were also reached in studies conducted on *Xenopus* oocytes (Andresson and Ruderman, 1998; Maton *et al*., 2003). Considering the complexity of AurA and CDK1 interaction, we have also addressed the issue of the dependence of AurA on CDK1 as prerequisite to the investigation of the pathways signaling DNA damage to AurA. We observed that treatment of synchronized HeLa cells with the selective cyclin-dependent kinase inhibitor roscovitine led to complete inhibition of CDK1, but left AurA activity unaffected. The drug neither impaired accumulation of AurA at centrosomes nor blocked centrosome separation, an event known to be under the control of AurA and Nek2 (Mayor *et al*., 1999; Nigg, 2001). These data demonstrated that in somatic cells, AurA activity was independent of CDK1 as early as 6 h postrelease from the G1/S block. These data allowed inferring that at the time of etoposide treatment (8 h postrelease) the inhibition of AurA activity that we observed occurred independently on effects of DNA damage on CDK1.

To address whether DNA damage-dependent AurA accumulation and inhibition of kinase activity was mediated by checkpoint kinases, we have taken a two-pronged approach: on the one hand we assessed the DNA damage response in HCT-15 cells, which express a form of Chk2 that does not respond to DNA damage. On the other hand, we employed siRNA to Chk1 or the potent Chk1 inhibitor UCN-01. The results showed that DNA damage was signaled to AurA in a Chk1-dependent manner. Identifying the Chk1 pathway as a transducer of DNA damage signals to AurA allowed us to definitely rule out unspecific stress responses as a cause of AurA inhibition.

We next addressed the mechanism of AurA inhibition by DNA damage. We excluded the possibility of tyrosine phosphorylation in the Gly-rich motif, which is the specific mechanism of CDK1 inactivation: AurA

Figure 6 Transduction of wt-AurA in etoposide-damaged cells promotes entry into mitosis. (**A**) Hydroxyurea block-released U2-OS cells were treated with etoposide at 8 h (a), nocodazole at the time of release (b) or nocodazole and etoposide (c). Cells treated with etoposide and nocodazole as in (c) were transduced at 10 h postrelease with vehicle peptide alone (d), wt-AurA (e) or kinase-dead AurA (f). Cells were analysed at 20 h by phase contrast microscopy. An average of 100–150 cells were counted in triplicate determinations. (**B**) Double-thymidine synchronized HeLa cells were treated with etoposide at 8 h (a), nocodazole at the time of release (b) or nocodazole and etoposide (c). Cells treated with etoposide and nocodazole as in (c) were transduced at 9 h postrelease with either vehicle peptide alone (d), wt-AurA (e) or wt-AurA in the presence of roscovitine (f), and analysed at 20 h by phase contrast microscopy. An average of 200–250 cells were counted in triplicate determinations. (**C**) Synchronized HeLa cells were damaged with etoposide, transduced with carrier peptide or wt-AurA and treated with nocodazole to prevent progression beyond M phase, as shown in **B**, panel e. Rounded-up cells (lane 2) were separated from adherent cells (lane 3) by mitotic shake-off. Phosphorylation of CDK1 Tyr_{15} as well as total CDK1 protein is shown. Lane 1: cells treated with vehicle peptide alone. (**D**) Double-thymidine synchronized HeLa cells were treated with etoposide at 8 h postrelease (a, b) and transduced with wt-AurA at 9 h in the absence (c, d) or the presence of roscovitine (e, f). Cells were fixed at 10 h and analysed by immunofluorescence using purified AurA antibody (a, c, e) or DAPI (b, d, f).

contains a phenylalanine at the position corresponding to Tyr_{15} in CDK1 and we were unable to detect phosphorylation at tyrosine residues in AurA upon damage (data not shown). Direct phosphorylation of AurA by checkpoint kinases was ruled out due to the inability of recombinant AurA to serve as *in vitro* substrate for either Chk1 or Chk2 that were immunoprecipitated from UV- or IR-treated cells, respectively (data not shown). Considering that phosphorylation has been implicated in the control of *X. laevis* AurA kinase activity, we have generated a nonphosphorylatable mutant of a site that was described as inhibitory in studies conducted on *X. laevis* AurA (Littlepage *et al.*, 2002). We found that the A_{342}-AurA mutant displayed an activity similar to the wt kinase and, most importantly, was resistant to inhibition by DNA damage. The last question that we have addressed was the impact of DNA damage-dependent AurA inhibition on the onset of mitosis. A previous study claimed that inducible expression of wt-AurA could overcome the slight reduction (25–30%) of mitotic entry observed in irradiated fibroblasts (Marumoto *et al.*, 2002). However, considering that overexpression of either wt- or kd-AurA was later shown to regulate equally well a number of mitotic events (Meraldi *et al.*, 2002), the possibility remained that the effect observed in irradiated fibroblasts might have occurred independently of AurA kinase activity. Moreover, since Marumoto *et al.* (2002) argued in their study that AurA was downstream of CDK1, the AurA-driven bypass of the DNA damage checkpoint and the following mitotic entry observed by the Authors were necessarily implied to occur in the absence of CDK1 activity. This is clearly incompatible with our current knowledge of mitotic regulation (Nigg, 2001). In the present study we unambiguously show that in HEK 293T cells that were arrested by DNA damage to a more significant extent than what was reported for fibroblasts, ectopic expression of wt, but not kinase-dead, AurA drove cells to mitosis. In this setting, a mutant of a site of phosphorylation that negatively affects AurA activity displayed a more pronounced bypass of the DNA damage-induced G2 arrest as compared to wt-AurA. Despite the fact that transient expression studies are widely used to assess the biological function of proteins, we did not regard this approach as sufficient to establish the role of AurA. We considered stable transfection of cells with inducible forms of AurA also not suitable, since the process of induction is normally not compatible with the fast kinetics that are the object of this study (i.e. generation of damage shortly before entry into mitosis). For this reason, we took an alternative approach by employing a technology that allows rapid and efficient transduction of proteins into cells based on the property of a peptide carrier (Morris *et al.*, 2001). Peptide-mediated protein transduction is a well-established technique (Joliot and Prochiantz, 2004), which is equivalent to microinjection, but shows the advantage of avoiding the stress associated with physical piercing of the membrane in target cells. The results obtained upon transduction of active, but not kinase-dead, AurA in various cell lines that were fully arrested by etoposide indicated that AurA was capable of advancing cells into mitosis, thus bypassing the G2 block. Of particular note is the fact that transduction of active AurA resulted in nuclear envelope breakdown and formation of the mitotic spindle, functions that are controlled by CDK1. Analysis of CDK1 status in transduced vs nontransduced cells, showed that DNA damage-inhibited CDK1 was reactivated in the former and, accordingly, treatment of AurA-transduced cells with roscovitine abolished entry into mitosis. This is the first formal demonstration that, in the context of DNA damage, AurA operates upstream of CDK1 and that AurA-mediated bypass of the DNA damage checkpoint proceeds through reactivation of CDK1. As shown in the model that summarizes our findings (Figure 7), reactivation of CDK1 by AurA could proceed via a positive input onto Cdc25 phosphatases or through downregulation of the inhibitory kinases Wee1 or Myt1. Whereas evidence is available for the former pathway (Dutertre *et al.*, 2004), the possible control of Wee1/Myt1 kinase activity by AurA has not been investigated to date. Thus, our data not only complete and extend previous observations, but also provide the correct interpretation

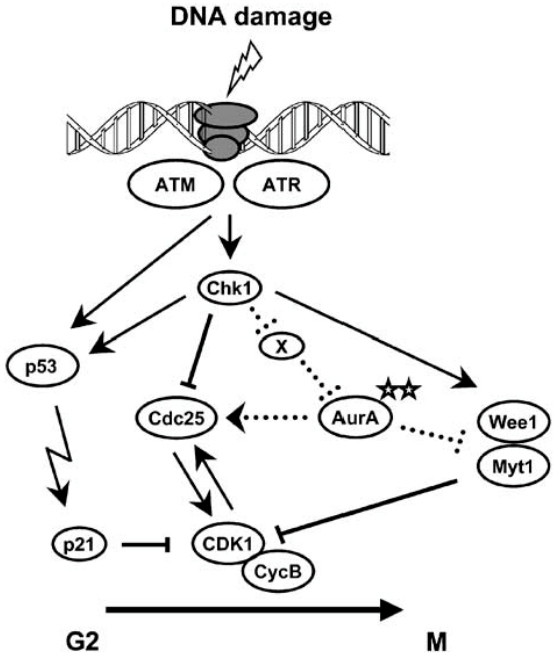


Figure 7 Model depicting the role of AurA at the onset of mitosis in DNA-damaged cells. Double-strand breaks (DSB) in DNA are recognized and initially processed by specific protein complexes (sensors, depicted in gray). As a result, structures are generated that allow binding and activation of signal transducers like ATM and ATR (Nyberg *et al.*, 2002). When DSB are generated during transition through G2, ATM/ATR-dependent activation of Chk1 results in inhibition of Cdc25 and AurA among others. This ultimately leads to CDK1 inhibition and cell cycle arrest. The presence of highly active AurA (indicated by the double asterisk) can promote constitutive activation of CDK1, either through a positive input onto Cdc25 phosphatases or through inhibition of Wee1/Myt1 activity, thus allowing bypass of DNA damage signals.

of the hierarchy of events occurring at mitosis. It will now be interesting to elucidate the pathway controlled by AurA and leading to CDK1 reactivation.

Acknowledgements

We thank F Forcellino for having made preliminary observations, J Jiricny, P Schaer and M El-Shemerly for critical reading of the manuscript and EA Nigg for helpful suggestions. We are also indebted to S Kleiner and Y Nagamine for help and suggestions in the siRNA experiments, JL Salisbury for centrin monoclonal antibody, A Kraemer and J Lukas for CHK1 monoclonal antibody and S Hausaman, Cancer Therapy Evaluation Program, NCI, NIH, Rockville, MD, USA, for supplying UCN-01. SF is supported by a grant of the Zurich Cancer League and CP is supported by the Ligue Nationale Contre le Cancer (équipe Labéllisée).

References

Andoh T, Ishida R. (1998). *Biochim Biophys Acta* **1400**: 155–171.
Andresson T, Ruderman JV. (1998). *EMBO J* **17**: 5627–5637.
Biggins S, Severin FF, Bhalla N, Sassoon I, Hyman AA, Murray AW. (1999). *Genes Dev* **13**: 532–544.
Bischoff JR, Anderson L, Zhu Y, Mossie K, Ng L, Souza B *et al.* (1998). *EMBO J* **17**: 3052–3065.
Castro A, Arlot-Bonnemains Y, Vigneron S, Labbe JC, Prigent C, Lorca T. (2002). *EMBO Rep.* **3**: 457–462.
Chan CS, Botstein D. (1993). *Genetics* **135**: 677–691.
Charrasse S, Carena I, Brondani V, Klempnauer KH, Ferrari S. (2000). *Oncogene* **19**: 2986–2995.
Crawford DF, Piwnica-Worms H. (2001). *J Biol Chem* **276**: 37166–37177.
Davies SP, Reddy H, Caivano M, Cohen P. (2000). *Biochem J* **351**: 95–105.
De Azevedo WF, Leclerc S, Meijer L, Havlicek L, Strnad M, Kim SH. (1997). *Eur J Biochem* **243**: 518–526.
Dutertre S, Cazales M, Quaranta M, Froment C, Trabut V, Dozier C *et al.* (2004). *J. Cell Sci* **117**: 2523–2531.
Falck J, Lukas C, Protopopova M, Lukas J, Selivanova G, Bartek J. (2001). *Oncogene* **20**: 5503–5510.
Ferrari S, Marin O, Pagano MA, Meggio F, Hess D, El-Shemerly M *et al.* (2005). *Biochem J* **390**: 293–302.
Giet R, Prigent C. (1999). *J Cell Sci* **112**(Part 21): 3591–3601.
Glover DM, Leibowitz MH, McLean DA, Parry H. (1995). *Cell* **81**: 95–105.
Haydon CE, Eyers PA, Aveline-Wolf LD, Resing KA, Maller JL, Ahn NG. (2003). *Mol Cell Proteomics* **2**: 1055–1067.
Hirota T, Kunitoku N, Sasayama T, Marumoto T, Zhang D, Nitta M *et al.* (2003). *Cell* **114**: 585–598.
Honda K, Mihara H, Kato Y, Yamaguchi A, Tanaka H, Yasuda H *et al.* (2000). *Oncogene* **19**: 2812–2819.
Joliot A, Prochiantz A. (2004). *Nat Cell Biol* **6**: 189–196.
Katayama H, Zhou H, Li Q, Tatsuka M, Sen S. (2001). *J Biol Chem* **276**: 46219–46224.
Kimura K, Hirano M, Kobayashi R, Hirano T. (1998). *Science* **282**: 487–490.
Lengauer C, Kinzler KW, Vogelstein B. (1998). *Nature* **396**: 643–649.
Littlepage LE, Ruderman JV. (2002). *Genes Dev* **16**: 2274–2285.
Littlepage LE, Wu H, Andresson T, Deanehan JK, Amundadottir LT, Ruderman JV. (2002). *Proc Natl Acad Sci USA* **99**: 15440–15445.
Loeb LA, Loeb KR, Anderson JP. (2003). *Proc Natl Acad Sci USA* **100**: 776–781.
Marumoto T, Hirota T, Morisaki T, Kunitoku N, Zhang D, Ichikawa Y *et al.* (2002). *Genes Cells* **7**: 1173–1182.
Marumoto T, Honda S, Hara T, Nitta M, Hirota T, Kohmura E *et al.* (2003). *J Biol Chem* **278**: 51786–51795.
Maton G, Thibier C, Castro A, Lorca T, Prigent C, Jessus C. (2003). *J Biol Chem* **278**: 21439–21449.
Mayor T, Meraldi P, Stierhof YD, Nigg EA, Fry AM. (1999). *FEBS Lett* **452**: 92–95.
Meraldi P, Honda R, Nigg EA. (2002). *EMBO J* **21**: 483–492.
Morris MC, Depollier J, Mery J, Heitz F, Divita G. (2001). *Nat Biotechnol* **19**: 1173–1176.
Nghiem P, Park PK, Kim Y, Vaziri C, Schreiber SL. (2001). *Proc Natl Acad Sci USA* **98**: 9092–9097.
Nigg EA. (2001). *Nat Rev Mol Cell Biol* **2**: 21–32.
Nyberg KA, Michelson RJ, Putnam CW, Weinert TA. (2002). *Ann Rev Genet* **36**: 617–656.
Peters JM. (2002). *Mol Cell* **9**: 931–943.
Rouse J, Jackson SP. (2002). *Science* **297**: 547–551.
Sen S, Zhou H, White RA. (1997). *Oncogene* **14**: 2195–2200.
Smits VA, Medema RH. (2001). *Biochim Biophys Acta* **1519**: 1–12.
Stenoien DL, Sen S, Mancini MA, Brinkley BR. (2003). *Cell Motil Cytoskeleton* **55**: 134–146.
Walter AO, Seghezzi W, Korver W, Sheung J, Lees E. (2000). *Oncogene* **19**: 4906–4916.
Zhao B, Bower MJ, McDevitt PJ, Zhao H, Davis ST, Johanson KO *et al.* (2002). *J Biol Chem* **277**: 46609–46615.
Zhou H, Kuang J, Zhong L, Kuo WL, Gray JW, Sahin A *et al.* (1998). *Nat Genet* **20**: 189–193.

Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).

2. MECHANISMS CONTROLLING AURORA A INHIBITION UPON DNA DAMAGE

AGNIESZKA KRYSTYNIAK and STEFANO FERRARI

Manuscript in preparation

Mechanisms controlling Aurora A inhibition upon DNA damage

Agnieszka Krystyniak and Stefano Ferrari*

Institute of Molecular Cancer Research
University of Zürich
Winterthurerstrasse 190
CH-8057 Zürich
Switzerland

*Corresponding author
Tel: +41-44-635-3471
Fax: +41-44-635-3484
E-mail: sferrari@imcr.unizh.ch

Abstract

Upon induction of DNA damage in form of double strand breaks several kinase pathways are activated leading to the cell cycle arrest. Among them Aurora A was found to be actively inactivated with corresponding lack of its degradation. Here, we provide evidence that inhibition of Aurora A kinase activity is dependent on its binding status to protein phosphatase 1 (PP1) and partially to TPX2. We demonstrate that upon etoposide treatment PP1 binding to Aurora A is diminished. Using mutants with point mutation in one of the sites responsible for PP1 binding (S342->A and S342->D) we show that phosphorylation at this residue plays a role in binding of PP1 both in the normal conditions and upon induction of double strand breaks. Lack of S342 phosphorylation abrogates releasing of PP1 from Aurora A – PP1 complex upon DNA damage. Our previous study showed that the same mutation (S342A) prevents Aurora A from deactivation in response to damage.

We also show that upon DNA damage TPX2 binding to Aurora A is highly impaired. On the other hand, the phosphorylation status of T288 – a T-loop site – does not change dramatically in the damage conditions. These findings indicate that inhibition of the kinase activity of Aurora A upon DNA damage is a complex process that requires orchestrated action of several factors and that S342 site plays there an important role.

Introduction

One of the most important features to the maintenance of genome stability is the ability of cells to respond to DNA damage by slowing down or temporarily blocking the cell cycle, thus allowing damage to be repaired. This process, known as "DNA damage response" involves sensing of damage by a specialized set of proteins, transmission of this signal to repair proteins and at the same time delaying the onset of mitosis, a process that is also under control of a specialized protein network. Defects in the so-called cell cycle checkpoint pathways are associated with an array of phenotypes in mammals including cancer predisposition and neurodegeneration.

The key components of mammalian DNA structure checkpoints' network can be divided into five categories, based on the position and general function in the network: (1) sensors – Rad9-Hus1-Rad1, so-called 9.1.1 complex, PCNA-like sliding clamp complex, the Rad17-RFC clamp loading complex and possibly the Mre11-Rad50-Nbs1 or MNR nuclease complex (Melo J. et al, 2002), (Petrini J.H. and Stracker T.H., 2003), (2) mediators – BRCA1, MDC1/NFBD1, 53BP1 and Claspin (Petrini J.H. and Stracker T.H., 2003), (3) signal transduction kinases – kinases of the phosphatidylinositol 3-kinase (PI3K)-like family, ATM and ATR (Abraham R.T., 2001), (Siloh Y., 2003) (4) effector kinases – Chk1 and Chk2 (Bartek J. and Lucas J., 2003) and (5) effector proteins – a large and diverse group encompassing cell cycle regulators such as the Cdc25 phosphatase, various DNA repair proteins, transcription factors such as p53 and E2F, chromatin components and regulators such as histone H2AX and Tlk kinases, and others (Zhou B.B. and Elledge S.J., 2000), (Donzelli M. and Draetta G.F., 2003).

Lately it has been also postulated that kinases involved in proper spindle formation play important roles in the cellular response to DNA damage, among them Plk1, Nek-2 and Aurora A.

Aurora A is a protein kinase possessing an important regulatory role in spindle formation, and chromosome segregation. Its level and activity are cell cycle regulated with a peak of both at mitosis and rapid degradation shortly thereafter (Bischoff J.R. et al, 1998), (Krystyniak A et al, 2006). Overexpression of Aurora A protein was found in several cancers, such as breast (Royce M.E. et al, 2003), colorectal (Bischoff J.R. et al, 1998), bladder (Tseng Y-S. et al, 2005), thyroid (Ulisse S. et al, 2006), NHL (Non-Hodgkin's

Lymphoma) (Yakushijin Y. et al, 2004) and gastric cancer (Yamada K.K. et al, 2004) in a manner that is both dependent and independent from gene amplification.

Aurora A activity is regulated both by its phosphorylation and interaction with other proteins. Among the most influencing interactors, working antagonistically to one another, are TPX2 and PP1. *In vitro*-dephosphorylated Aurora A, becomes phosphorylated and activated by TPX2 in an ATP-dependent manner (Eyers P.A. et al, 2003), in a process that requires its own kinase activity. TPX2 therefore stimulates autophosphorylation and autoactivation of the enzyme by strictly mechanistic protection of the T-loop site from dephosphorylation by PP1 (Bayliss R. et al, 2003). Three phosphorylation sites have been found in mitotic Aurora A. Threonine 288, localized in the activation loop of the catalytic domain of the kinase is necessary for the kinase activity. Although this residue can be phosphorylated *in vitro* by protein kinase A (PKA) (Walter A.O. et al, 2000), a role for PKA *in vivo* is hardly possible, since PKA activity is low when cells approach mitosis. On the other hand, Aurora A was shown to be able to autophosphorylate at a number of sites, including Threonine 288 and thereby autoactivate (Ferrari S. et al, 2005). Two other phosphorylation sites have been found in *Xenopus* Aurora A, namely Serine 53 and Serine 349 (S51 and S342 in the human Aurora A) (Littlepage L.E. et al, 2002). S53 is part of the A-box required for destruction of Aurora A at mitosis, whereas S349 is adjacent to one of the protein phosphatase 1 (PP1)-binding motifs. Mutations of Serine 53 to both Alanine and Aspartic acid had no significant effect on kinase activity. However, the Asp53 was shown to be more stable than wild-type, suggesting that phosphorylation at this site might regulate Aurora A destruction during mitosis (Littlepage L.E. et al, 2002). Mutation of Serine 349 to Alanine slightly reduced Aurora A activity, whereas mutation of this site to Aspartic acid completely abolished it. The Authors suggested that either phosphorylation of Serine 342 is inhibitory, or that it is critical for the regulation of Aurora A in some other way. Our data indicates that mutation of S342 to Alanine in human Aurora A results in an isoform displaying very similar activity to the wild type, which is, more importantly, not inhibited upon DNA damage (Krystyniak A. et al, 2006). Therefore we set out to investigate the importance of this site in Aurora A response to double-strand breaks introduced to DNA. We show that upon DNA damage PP1 is released from Aurora A and that this process is under control of phosphorylation status of S342. TPX2 is also partially separated from Aurora A upon etoposide treatment, a process that should impair the protection of T288 from dephosphorylation. On the other hand, the phosphorylation status of T288 remains unchanged, namely similar to that observed during transition through

mitosis, at least at early times after the induction of damage. Those results point to a far more complex regulation of Aurora A activity than it has been so far postulated and, for the first time, prove that S342 is indeed a site responsible for negative regulation of Aurora A.

Results and Discussion

PP1 binding to Aurora A is abolished upon DNA damage

Activated Aurora A is known to phosphorylate PP1 and therefore inhibit its activity *in vitro* as well as *in vivo*, although the evidence for direct phosphorylation is not available to date. On the other hand, PP1 was shown to dephosphorylate active Aurora A, and thereby abolish its activity *in vitro*. Considering our findings that Aurora A is inhibited in response to DNA damage, we set out to investigate the binding of PP1 to Aurora A in such conditions. We used etoposide, the topoisomerase II inhibitor, to create double-strand breaks in DNA after completion of its synthesis, namely 8 hours post-release from second thymidine block, when Aurora A level, activity as well as its PP1 binding is close to maximum. As shown previously (Krystyniak A. et al, 2006), etoposide-induced DNA damage leads to block of the cell cycle in G2, accumulation of Aurora A protein and inhibition of its kinase activity. In such conditions PP1 binding to Aurora A is highly decreased (Figure 1A). Treatment of cells with okadaic acid, a known inhibitor of both PP1 and PP2A, resulted in partial, though reproducible, decrease in binding of Aurora to PP1. This indicates that binding of PP1 to Aurora A may partially depend on enzymatic activity of PP1 (Figure 1B).

PP1 binding depends on S342 phosphorylation status

Aurora A in known to posses two PP1-binding motifs, one that includes the catalytic lysine residue (K^{169}VLF) and a second that is immediately adjacent to Serine 342 (K^{343}VEF) (Katayama H. et al, 2001). The results above led us to formulate the hypothesis that phosphorylation of Aurora A at Serine 342 may alter binding of PP1 at the adjacent K^{343}VEF motif, an event that may affect phosphorylation at the T-loop Threonine 288 as well as at other sites required for Aurora A activity (Littlepage L.E. et al, 2002). To test this hypothesis, we transfected Aurora A wild-type, as well as the non-phosphorylable S342A mutant or the "constitutively phosphorylated" S342D mutant (with Aspartic acid mimicking constitutive phosphorylation) into HEK-293T cells, between two thymidine

blocks. After release from the second Thymidine block and completion of DNA synthesis, cells were treated with etoposide to create double-strand breaks, and harvested at the time when they were supposed to have reached mitosis (6,5 hours for HEK-293T cells). Immunoprecipitation using polyclonal anti-Aurora A antibody (PAb-36) brought down both endogenous Aurora A together with recombinant wild-type or mutant kinase, as well as its interacting partner PP1.

In mitotic cells (Figure 2B), the binding of PP1 to Aurora A appeared to be slightly lower in case of the S342A mutant (lane 3), compared to the wild-type (lane 2), and highly impaired in case of S342D mutant (lane 4) – the level of PP1 in this case is similar to that in the mock-transfected sample (lane 1). The small amount of PP1 co-immunoprecipitating with Aurora A in lane 1 is likely attributable to interaction with the endogenous kinase. Kinase-dead Aurora A mutant (mutation D145N), on the other hand, showed the greatest binding to PP1 (data not shown), supporting the hypothesis that Aurora A activity may play a role in controlling the extent of PP1 binding. Binding of wild type Aurora A to PP1 was highly decreased upon DNA damage (compare lanes 2 with 6). In contrast, in case of S342A Aurora A there was only a slight decrease observed (compare lane 3 to 7).

This data show that binding of PP1 to Aurora A depends on the phosphorylation status of Aurora A on Serine 342 and that, upon DNA damage, impaired phosphorylation at this site significantly prevents PP1 from dissociating from the complex.

Wild-type and S342A mutant Aurora A, but not S342D mutant, can drive cells to mitosis

The evidence presented above, together with previously published data (Krystyniak A. et al, 2006) that S342A Aurora A is resistant to DNA damage-induced inhibition and that introduction of this mutants into DNA-damaged cells causes even greater by-pass of the G2 arrest than wild-type, suggests that Serine 342 is a negative regulatory site for the activity of Aurora A. To further prove this hypothesis, transduction experiments in U2OS and HEK-293T cells using both AurA S342A and S342D mutants, were performed. Both FACS and immunofluorescence analysis of HEK-293T cells, providing a read-out for the cell cycle distribution and the presence of mitotic figures, respectively, showed that AurA wt and S342A mutant, but not S342D mutant, have the ability to push cells towards mitosis, despite the presence of DNA damage (Figure 3A and B). Western blot analysis, using as mitotic marker phosphorylated-H3, showed a signal only in extracts from cells

transduced with wild-type and S324A AurA (lanes 3 and 4, Figure 3C, respectively). No histone phosphorylation could be observed in control extracts from cell treated with etoposide and nocodazole (lane 1), as well as in extracts from cells transduced with S342D AurA (lane 5).

TPX2 binding to Aurora A is decreases upon DNA damage

TPX2 (Target Protein for *Xenopus* kinesin-like protein 2) is a microtubule-associated protein, which is required for induction of spindle assembly (Gruss O.J. et al, 2002). It has been shown that NH_2 terminus of TPX2 binds to COOH-terminal catalytic domain of Aurora A, and targets it to the spindle apparatus (Kufer T.A. et al, 2002), namely spindle microtubules. Since we have previously shown that Aurora A is inhibited upon DNA damage, we set out to investigate whether there is any correlations between loss of kinase activity and disruption of the TPX2-Aurora A complex upon induction of double-strand breaks. To this end, we immunoprecipitated Aurora A from cells synchronized at the G1/S boarder (non-released from double Thymidine block), cells in M phase (10 hr from the release from the second Thymidine block) or cells treated with etoposide in the G2 phase of the cell cycle (8 hr) and harvested at the time corresponding to transition through M phase (10 hr from the release from the second Thymidine block) using polyclonal antibody PAb-36. The data showed that Aurora A protein co-immunoprecipitated with its partner TPX2. Aurora A level in G1/S cells was low (Figure 3B, lane 1), so non-surprisingly also its binding partner's level was hardly detectable (Figure 3B, lane 5). Since TPX2 is known to be cell cycle regulated (Steward S. and Fang G., 2005), the low level of protein in G1/S cells was expected (Figure 3A, lane 1). On the contrary, the level of both proteins as well as their interaction increased and reach maximum in M phase (Figure 3B, lanes 2 and 6). Upon DNA damage, however, despite the level of both proteins was as high as in mitosis (Figure 3B, lane 3), interaction between them seemed to be significantly impaired, as indicated by the decrease in the co-immunoprecipitated TPX2 (Figure 3B, lane 7).
Examination of the Aurora A and TPX2 distribution in the cells by immunofluorescence showed perfect co-localization in mitotic cells (Figure 3A, middle panel and Figure C, top panel). This is consistent with the known fact that TPX2 is involved in proper localization of Aurora A to the spindle poles and spindle microtubules (Kufer T.A. et al, 2002). Analysis of the time course co-localization of these two proteins revealed that at 8 hours

post-release Aurora A did not co-localize with TPX2, especially at centrosomes, suggesting that the interaction is tightly cell cycle regulated and restricted to the mitotic division. Late time points of mitosis showed partial loss of co-localization, with Aurora A being more abundant at centrosomes and TPX2 in the mitotic bridge.

We have previously shown that, despite the presence of DNA damage, Aurora A is able to partially localize to the centrosomes, (Krystyniak A. et al, 2006). However, co-localization with TPX2 at centrosomes was not observed any longer under these conditions (Figure 3C, bottom panel and lower pictures of each panel of Figure 3A). TPX2 rather displayed diffused nuclear staining, which overlapped with the staining of Aurora A. This explains the residual interaction between the two proteins found in co-immunoprecipitation experiments, which seems at this point to be rather "physical" then "functional".

T288 phosphorylation status does not change upon DNA damage

Similarly to a number of other kinases Aurora A activity is regulated by phosphorylation within the activation loop of the catalytic domain of the kinase, namely the conserved residue Threonine 288. Considering that phosphorylation at this residue results in a significant increase of Aurora A kinase activity, we decided to investigate the phosphorylation status of Aurora A's Threonine 288 upon DNA damage. Western blot analysis using a specific antibody able to recognize phospho-Threonine 288, showed clear accumulation of phosphorylated Aurora A after treatment with okadaic acid (Figure 5A). This result was expected, given that inhibition of the two major phosphatase activities in the cell would tilt the balance toward phosphorylation, thus increasing the signal for phospho-Threonine 288 to a level higher than the "physiological" one observed in mitotic cells (Figure 5A, lanes 3 and 5 vs. lane 2). Surprisingly Aurora A remained phosphorylated at its activation loop after administration of etoposide (Figure 5B, lane 3), despite of the clear loss of kinase activity in these conditions (Krystyniak et al, 2006). Immunofluorescence examination of the cells showed strong staining with the antibody to phospho-Threonine 288 in cells analyzed at 10h post-release (Figure 5C, top panel), only in mitotic cells (as judged by chromosome condensation in DAPI labeled cells). In contrast cells treated with etoposide display quite strong, although diffused, staining in nearly all cells, without any signs of DNA condensation, or even centrosome separation (as a sign of Aurora A being active) (Figure 5C, bottom panel). This data goes in agreement with the

experiments done using crystal structures of Aurora A with or without its binding partner TPX2, which suggested that phosphorylation in the activation loop alone is not sufficient for enzyme activation (Eyers P.A. et al, 2005).

Materials and methods

Expression vectors, chemicals, and antibodies

Full-length Aurora A was obtained in PCR reaction as described previously (Krystyniak A. et al, 2006). The D145N, S342A and S342D mutants were generated using the QuickChange site-directed mutagenesis kit (Stratagene). Expression vectors for myc-tagged forms of wild type and kinase-dead (D145N) Aurora a in pCS2 were kindly provided by P. Sassone-Corsi (Strasbourg, France).

Recombinant proteins were generated by subcloning Aurora A ORF into pTXB3 vector (New England BioLabs) followed by expression of the intein-tagged fusion proteins in the BL21 *Escherichia coli* strain and purification using chitin beads.

A polyclonal serum to Aurora A was generated as described (Krystyniak A. et al, 2006) and monoclonal antibody as described (reference!). A polyclonal antibody recognizing phosphorylated at T288 Aurora A was obtained from BioLegend. A monoclonal antibody to TPX2 was kindly provided by Dr Hans-Jürgen Heidebrecht. A monoclonal antibody to protein phosphatase 1 was obtained from Lab Force.

The carrier peptide Pep-1 (Morris et al., 2001) was synthesized on a Milligen 9050 Plus automated peptide synthesizer (continuous flow) using chemical protocols based on Fmoc chemistry. The purity of the final compound was verified by reversed-phase analytical HPLC and the identity were assessed by correct mass spectral and amino acid analyses.

Cell culture, synchronization, transfection and transduction

HeLa and HEK-293T cells were maintained in DMEM (OmniLab) medium supplemented with 10% fetal calf serum (FCS, Life Technologies), penicillin (100U/ml) and streptomycin (100µl/ml). For synchronization experiments cells were treated with double thymidine block as described (Krystyniak A. et al, 2006).

Transfections were done using Metafectene (Biontex) according to the manufacturer's instructions.

Transductions were done using the carrier peptide Pep-1 according to the procedure described (Morris et al., 2001). Recombinant, purified proteins of Aurora A wild type and mutants S342A and S342D were used.

Immunofluorescence

Indirect immunofluorescence experiments were performed with cells grown on acid-washed glass cover slips as described (Charrasse *et al.*, 2000). Detection of AurA, P288-AurA and TPX2 were performed with purified AurA-Pab36 polyclonal antibody (1/800), anti-T288-AurA polyclonal antibody (1/50) and monoclonal antibody to TPX2 (1/1), respectively. FITC-labelled (1/750), TRITC-labelled (1/50) or Texas Red-labelled (1/200) secondary antibodies were combined with DAPI (Molecular Probes). Cells were observed with a Leica DMRB microscope equipped with a 100W HBO lamp for fluorescence. High-resolution pictures were taken with oil-immersion lenses (PL-FLUOTAR 40x-100x) and images were captured with a Leica DC 200 camera. Cells were viewed using Leica DC Viewer software and image merging was obtained using Adobe Photoshop 7.0.

Western Blotting and Immunoprecipitation

Cell extraction and detection of proteins by Western blot analysis was carried out as previously described (Charrasse *et al.*, 2000). Immunoprecipitations were carried out for 3 h at 4^{o}C in Buffer A (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 6 mM EGTA, 15 mM Na-pyrophosphate, 0.5 mM Na-orthovanadate, 1 mM benzamidine, 0.1 mM phenylmethylsulfonil fluoride (PMSF), 1% Nonidet P-40) using 100, 200 or 500 μg of total protein for CDK1, Chk2 or AurA, respectively. Proteins were immobilized on Protein A/G-Agarose beads (S. Cruz Biotech.) and washed in 3x 1 ml ice-cold Buffer A. AurA was routinely immunoprecipitated with purified AurA-Pab36 antibody and detected by Western blot with monoclonal antibody 35C1.

FACS and cell cycle analysis

HEK-293T cells, synchronized by double thymidine block were treated with etoposide at 4h post-release and half an hour later transduced with either wild type Aurora A or one of the S342 mutants (A or D). After 24h from release cells were trypsinized, washed with PBS and fixed with ice-cold ethanol for 12 hours in 4^0C. Fixed cells were treated for 30 min with RNase A (100 μg/ml) (Sigma) at 37^0C, DNA was labeled with propidium iodide (PI) (20 μg/ml) (Sigma). Analysis was performed using FC500 cytometer (Beckman Coulter).

References

Abraham R.T. "Cell cycle checkpoint signaling through the ATM and ATR kinases", 2001, *Genes and Development*; 15:2177-2196

Ahonen L.J., Kallio M.J., Daum J.R., Bolton M., Manke I.A., Yaffe M.B., Stukenberg P.T., Gorbsky G.J. "Polo-like kinase 1 creates the tension-sensing 3F3/2 phosphoepitope and modulates the association of spindle-checkpoint proteins at kinetochores", 2005, *Current Opinion in Biology*; 15:1078-1089

Bartek J., Lukas J. "Chk1 and Chk2 kinases in checkpoint control and cancer", 2003, *Cancer Cell*; 3:421-429

Bayliss R., Sardon T., Ebert J., Lindner D., Vernos I., Conti E. "Determinants for Aurora A and Aurora B discrimination by TPX2", 2004, *Cell Cycle*; 3:404-407

Bischoff J.R., Anderson L., Zhu Y., Mossie K., Ng L., Souza B., Schryver B., Flanagan P., Clairvoyant F., Ginther C., Chan C.S.M., Novotny M., Slamon D.J., Plowman G.D. "A homologue of Drosophila aurora kinase is oncogenic and amplified in human colorectal cancers", 1998, *The EMBO Journal*; 17:3052-3065

Donzelli M., Draetta G.F. "Regulating mammalian checkpoints through Cdc25 inactivation", 2003, *EMBO Reports*; 4:671-677

Eyers P.A., Erikson E., Chen L.G., Maller J.L. "A novel mechanism for activation of the protein kinase Aurora A", 2003, *Current Biology*, 13:691-697

Ferrari S., Marin O., Pagano M.A., Meggio F., Hess D., El-Shemerly M., Krystyniak A., Pinna L.A. "Aurora A site specificity: a study with synthetic peptide substrates", 2005, *Biochemical Journal*; 390:293-302

Fletcher L., Cerniglia G.J., Nigg E.A, Yend T.J., Muschel R.J. "Inhibition of centrosome separation after DNA damage: a role for Nek2", 2004, *Radiat Research*; 162:128-135

Fry A.M., Schultz S.J., Bartek J., Nigg E.A. "Substrate specificity and cell cycle regulation on Nek2 protein kinase, a potential human homolog of the mitotic regulator NIMA of Aspergillus nidulans", 1995, *Journal of Biological Chemistry*; 270:12899-12905

Goto H., Kiyono T., Tomono Y., Kawajiri A., Urano T., Furukawa K., Nigg E.A., Inagaki M. "Complex formation of Plk1 and INCENP required for metaphase-anaphase transition", 2005, *Nature Cell Biology*; 22:1676-1687

Krystyniak A., Garcia-Echeverria C., Prigent C., Ferrari S. "Inhibition of Aurora A in reponse to DNA damage", 2006, *Oncogene*; 1-11

Littlepage J.E., Ruderman J.V. "Identification of a new APC/C recognition domain, the A-box, which is required for the Cdh1-dependent destruction of the kinase Aurora A during mitotic exit", 2002, *Genes and Development*; 16:2274-2285

Littlepage J.E., Wu H., Andersson T., Deanehan J.K., Amundadottir L.T., Ruderman J.V. "Identification of phosphorylated residues that affect the activity of the mitotic kinase Aurora A", 2002, *PNAS*; 1-6

Melo J., Toczyski A. "A unified view of the DNA-damage checkpoint", 2002, *Current Opinion in Cell Biology*; 14:237-245

Meraldi P., Nigg E.A. “Centrosome cohesion is regulated by a balance of kinase and phosphatase activities”, 2001, *Journal of Cell Science*; 114:3749-3757

Petrini J.H., Stracker T.H. "The cellular response to DNA double-strand breaks: defining the sensors and mediators", 2003, *Trends in Cell Biology*; 13:458-462

Royce M.E., Xia W., Sahin A.A., Katayama H., Johnson D.A., Hortobagyi G., Sen S., Hung M-C. “STK15/Aurora A expression in primary breast tumors is correlated with nuclear grade but not with prognosis”, 2003, *Cancer*; 100;12-19

Siloh Y. "ATM and related protein kinases: safeguarding genome integrity", 2003, *Nature Reviews Cancer*; 3:155-168

Smits V.A.J., Klompmaker R., Arnaud L., Rijksen G., Nigg E.A., Medema R. “Polo-like kinase-1 is a target of the DNA damage checkpoint”, 2000, *Nature Cell Biology*; 2:627-676

Sumara I., Gimenez-Abian J.F., Gerlich D., Hirota T., Kraft C., de la Torre C., Ellenberg J, Peters J.M. “Roles of polo-like kinase 1 in the assembly of functional mitotic spindles”, 2004, *Current Opinion in Biology*; 14:1712-1722

Tseng Y-S., Tzeng C.C., Huang C-Y., Chen P-H., Chiu A. W-H., Hsu P-Y., Huang G-C., Wang Y-C., Liu H-S. “Aurora A overexpression associates with Ha-ras codon-12 mutation and blackfoot disease endemic area in bladder cancer”, 2005, *Cancer Letters*, 1-9

Ulisse S., Delcros J-G., Baldini E., Toller M., Curcio F., Giacomeli L., Prigent C., Ambesi-Impiombato F.S., D’Armiento M., Arlot-Bennemains Y. “Expression of Aurora kinases in human thyroid carcinoma cell lines and tissues”, 2006, *International Journal of Cancer*; published on-line 13.02.2006

van Vugt M.A.T.M., Bras A., Medema R.H. “Polo-like kinase-1 controls recovery from a G2 damage-induced arrest in mammalian cells”, 2004, *Molecular Cell*; 15:799-811

van Vugt M.A.T.M., Bras A., Medema R.H. “Restarting the cell cycle when the chceckpoint comes to a halt”, 2005, *Cancer Research*; 65(16):7037-7040

Walter A.O., Seghezzi W., Korver W., Sheung J., Lees E. “The mitotic serine/threonine kinase Aurora 2/AIK is regulated by phosphorylation and degradation”, 2000, *Oncogene*; 19:4906-4916

Yakushijin Y., Hamada M., Yasukawa M. “The expression of the Aurora A gene and its significance with tumorigenesis in Non-Hodgkin’s lymphoma”, 2004, *Leukemia and Lymphoma*; 45:1741-1746

Zhou B.B., Elledge S.J. "The DNA damage response: putting checkpoints in perspective", 2000, *Nature*; 408:433-439

Figure legends

Figure 1. Damage to DNA affects PP1 binding to Aurora A

A. (Left panel) Detection of Aurora A and PP1 by Western blot analysis of whole-cell extracts (WCE) derived from double-thymidine synchronized HeLa cells; NR (non-released) corresponds to cells in G1/S transition, 10h to mitotic cells, eto to cells treated with etoposide at 8h from the time of release from second thymidine block and harvested at 10h. (Right panel) Immunoprecipitation of 1 mg of the same WCE using anti-Aurora A polyclonal antibody followed by detection of precipitated Aurora A with its monoclonal antibody and detection of co-immunoprecipitated PP1 by Western blot.
B. (Left panel) Detection of Aurora A and PP1 by Western blot of WCE derived from synchronized HeLa cells. 0 corresponds to cells in G1/S, 10h to mitotic cells. Cells were treated with etoposide as described above and with okadaic acid at 7,5h from the release from the second thymidine treatment. (Right panel) Immunoprecipitation of 1 mg of the same WCE and detection by Western blot as described above.

Figure 2. Phosphorylation status of serine 342 plays a role in PP1 binding to Aurora A upon DNA damage.

A. 293T cells were synchronized by double-thymidine block and transfected with various Aurora A constructs in between the two blocks. 1 mg of DNA was introduced to the cells of either pcDNA3.1 vector (mock), Aurora A wild type (AurAwt), Aurora A point-mutated at D145->N (AurAkd), Aurora A point-mutated at S342->A (AurAS342A), Aurora A point-mutated at S342->D (AurAS342D) or Aurora A point-mutated at S51->D (AurAS51D). Cells were either collected at 8h (time corresponding to mitosis in 293T cells) (left) or treated with etoposide at 4h from the second release from the thymidine and harvested at 8h (right). Western blot analysis of 10 μg of WCE followed by detection of Aurora A, both endogenous and recombinant, and PP1.

B. Immunoprecipitation of 1 mg of above described WCE with anti-Aurora A polyclonal antibody followed by detection of Aurora A and PP1 by Western blot.

Figure 3. Transduction of wt-AurA in etoposide-damaged cells promotes entry into mitosis.

A. Double thymidine block-released HEK-293T cells were treated left untreated (NT), or treated with etoposide at 4 h (eto). Cells treated with etoposide as in (eto) were transduced at 4,5 h post-release with wt-AurA (AurAwt, eto), or AurA S342A (AurA342A, eto) or AurA S342D (AurA342D, eto). Cells were analyzed at 8 h by immunofluorescence using anti-AurA polyclonal antibody.
B. Double-thymidine synchronized HEK-293T cells were treated with etoposide and transduced as in panel A. Cells were harvested at 24h after release from the second thymidine block and subjected to FACS analysis.
C. Double thymidine block-released U2-OS cells were treated with etoposide at 8 h and nocodazole at 9h or with nocodazole alone. Cells treated with etoposide and nocodazole were transduced at 10 h post-release with wt-AurA, or AurA S342A or AurA S342D. Cells were harvested after 24 hours post-release and protein extracts analyzed by Western blot, using anti-H3 antibody for detection of mitotic cells. PP1 detection serves as loading control.

Figure 4. Binding of TPX2 to Aurora A is impaired upon DNA damage

A. (Left panel) Detection of Aurora A and TPX2 by Western blot analysis of whole-cell extracts (WCE) derived from double-thymidine synchronized HeLa cells; NR (non-released) corresponds to cells in G1/S transition, 10h to mitotic cells, eto to cells treated with etoposide at 8h and OA to cells treated with okadaic acid at 7,5h from the time of release from second thymidine block and harvested at 10h. (Right panel) Immunoprecipitation of 1 mg of the same WCE using anti-Aurora A polyclonal antibody followed by detection of precipitated Aurora A with its monoclonal antibody and detection of co-immunoprecipitated TPX2 by Western blot.

B. Immunofluorescence analysis of time-course of HeLa cells, synchronized by double thymidine block. Details of the procedure in **Materials and methods**
C. Immunofluorescence analysis of mitotic HeLa cells (control) and cells treated with etoposide at 8h from the release from the second thymidine block and analyzed at 10h (time corresponding to mitosis). Magnification of single cell showed.

Figure 5. Phosphorylation status of T288 is not affected upon DNA damage

A. HeLa cells were synchronized by double-thymidine block and treated with or without okadaic acid at 7,5h from the time of the second release. Cells were harvested at indicated time points and the total level of Aurora A protein as well as the level of phosphorylated at T288 fraction was analyzed by Western blot, using polyclonal anti-Aurora A antibody and specific antibody recognizing Aurora A phosphorylated at T288. Beta-tubulin serves as a loading control.
B. Detection of Aurora A total protein and Aurora A fraction phosphorylated at T288 by Western blot analysis of whole-cell extracts (WCE) derived from double-thymidine synchronized HeLa cells; NR (non-released) corresponds to cells in G1/S transition, 10h to mitotic cells, eto to cells treated with etoposide at 8h and OA to cells treated with okadaic acid at 7,5h from the time of release from second thymidine block and harvested at 10h. Beta-tubulin serves as a loading control.
C. Immunofluorescence analysis of Aurora A phosphorylated at position T288. Mitotic cells at the top panel, cells treated with etoposide at the bottom panel.

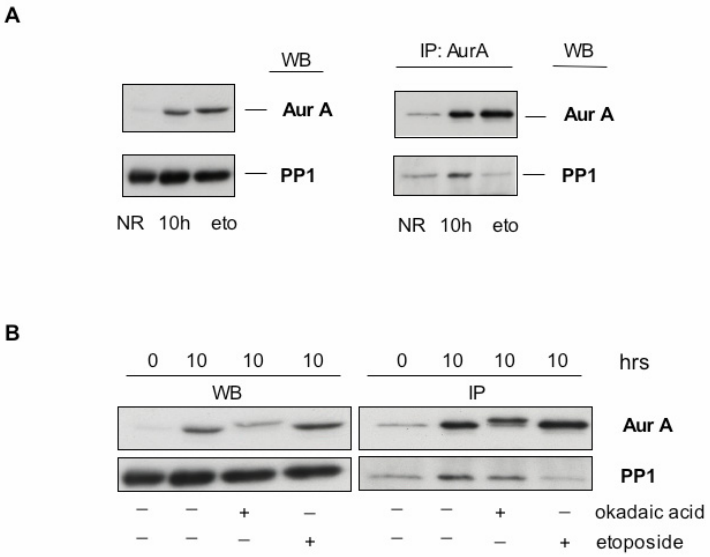


Figure 1. Krystyniak et al.

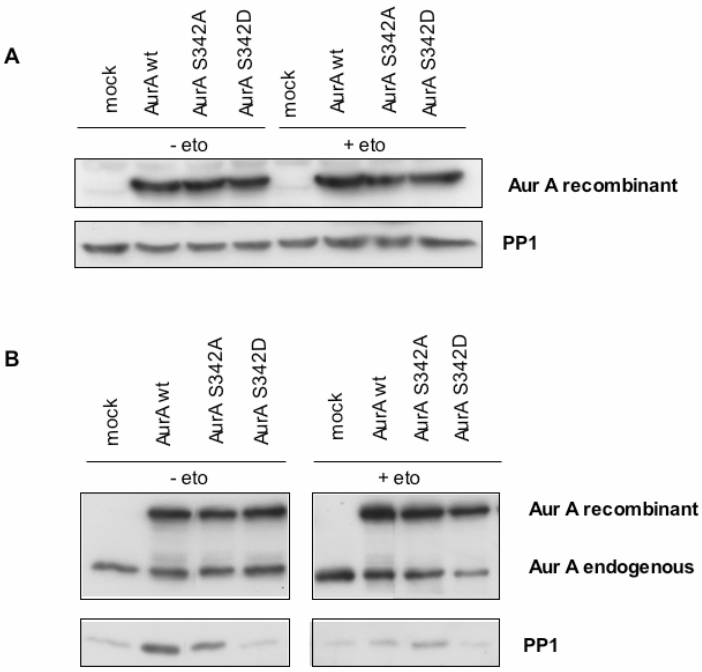


Figure 2. Krystyniak et al.

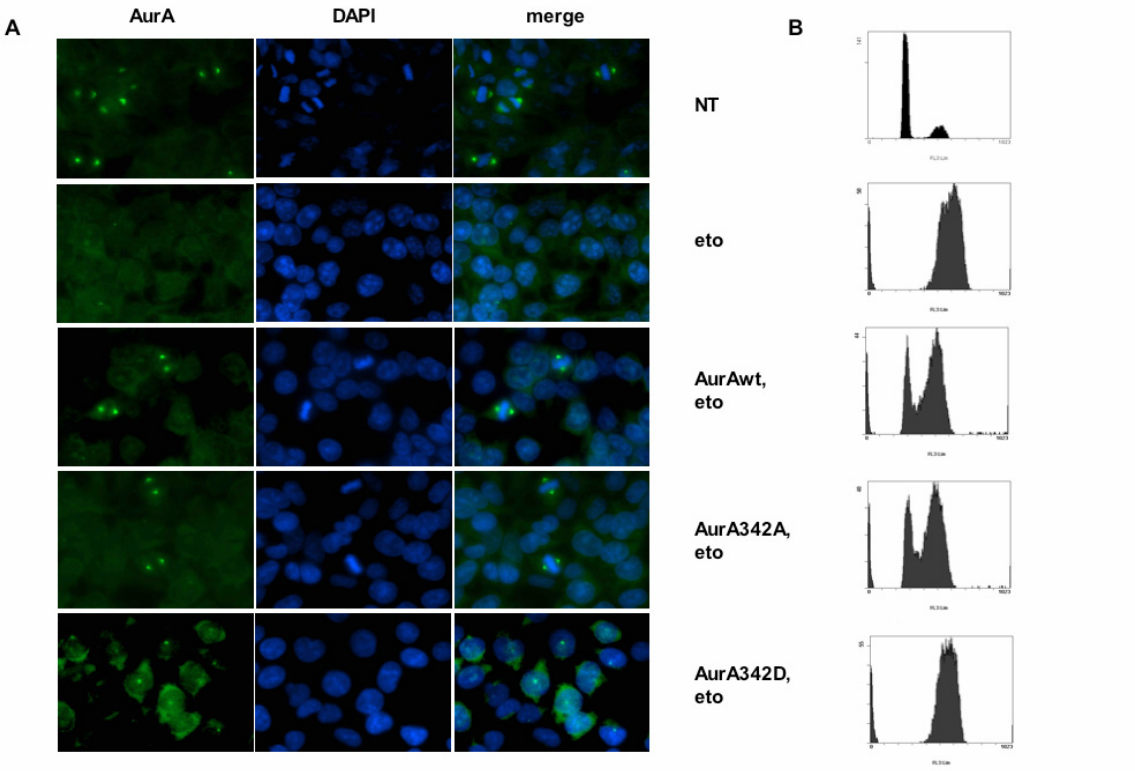


Figure 3. Krystyniak et al.

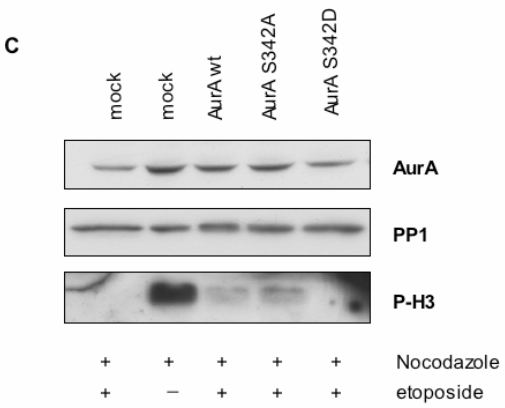


Figure 3. Krystyniak et al.

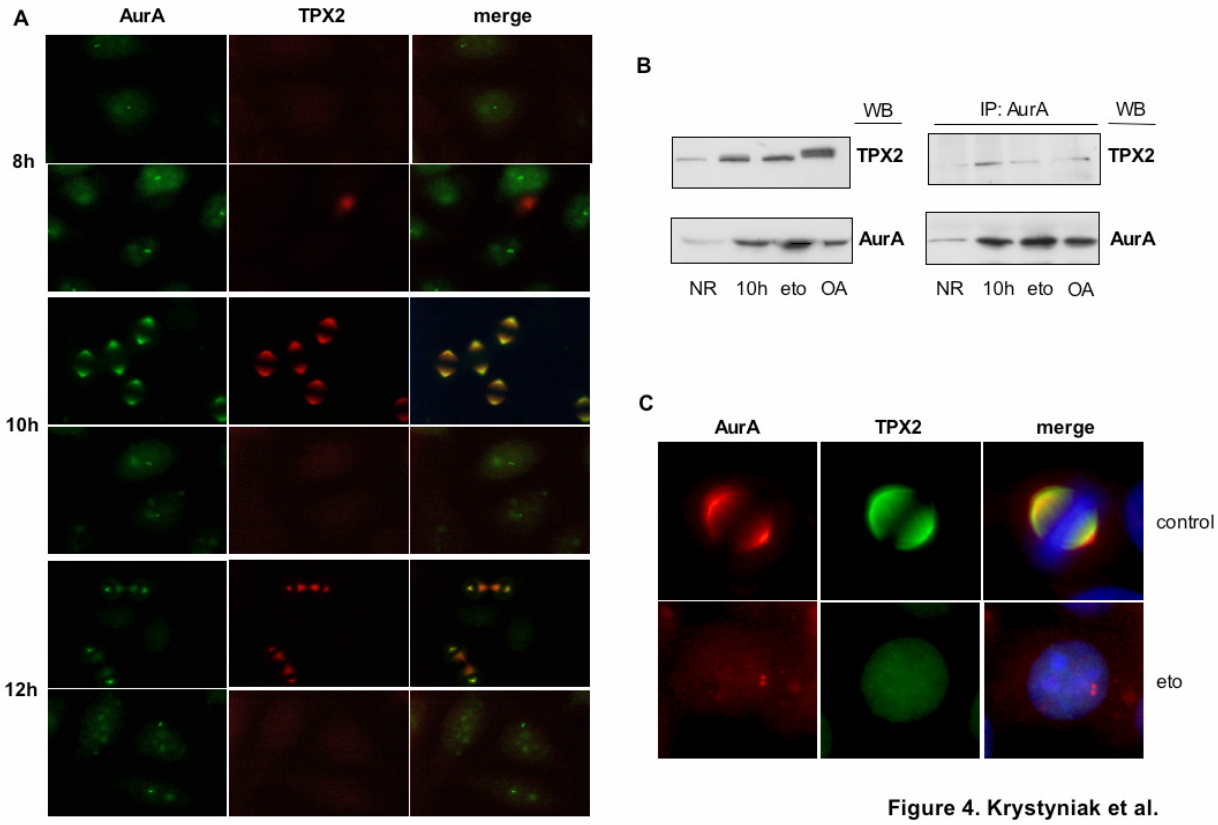


Figure 4. Krystyniak et al.

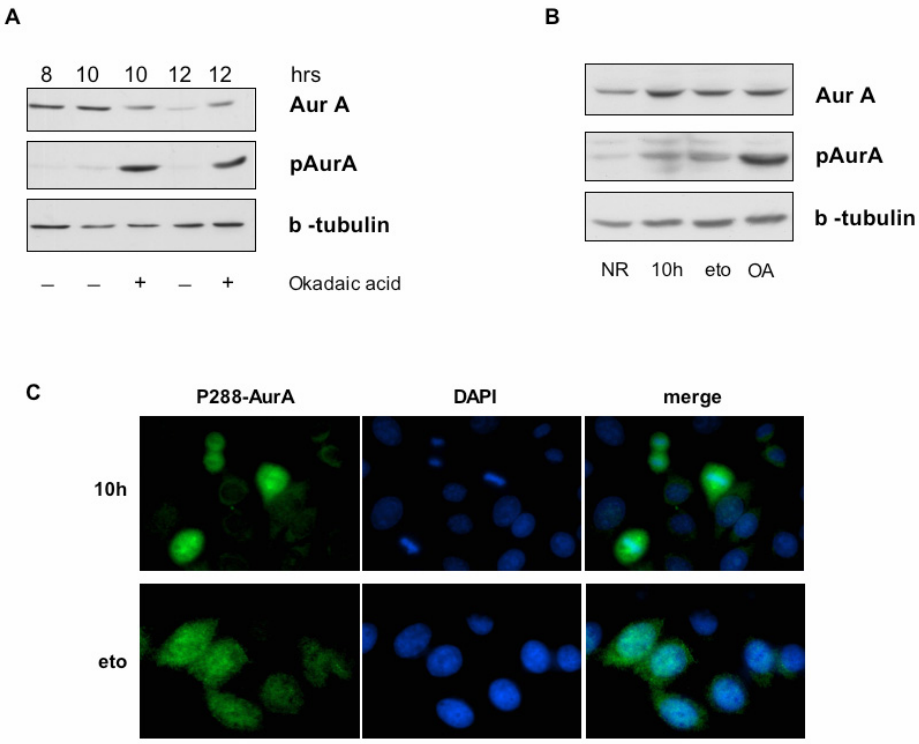


Figure 5. Krystyniak et al.

3. AURORA A SITE SPECIFICITY: A STUDY WITH SYNTHETIC PEPTIDE SUBSTRATES

S. FERRARI, O. MARIN, M.A. PAGANO, F. MEGGIO, D. HESS, M. EL-SHEMERLY, **AGNIESZKA KRYSTYNIAK** and L. PINNA

Reprinted from: *Biochemical Journal, 2005, Aug. 15; 390 (Pt 1): 293-302*

Biochem. J. (2005) **390**, 293–302 (Printed in Great Britain) doi:10.1042/BJ20050343

Aurora-A site specificity: a study with synthetic peptide substrates

Stefano FERRARI*[1], Oriano MARIN†‡, Mario A. PAGANO†, Flavio MEGGIO†, Daniel HESS§, Mahmoud EL-SHEMERLY*, Agnieszka KRYSTYNIAK* and Lorenzo A. PINNA†‡[1]

*Institute of Molecular Cancer Research, University of Zurich, Winterthurerstr. 190, CH-8057 Zurich, Switzerland, †Department of Biological Chemistry, University of Padova, Viale G. Colombo 3, I-35121 Padova, Italy, ‡Venetian Institute for Molecular Medicine, Via Orus 2, I-35129 Padova, Italy, and §Friedrich Miescher Institute, Maulbeerstrasse 66, CH-4058 Basel, Switzerland

AurA (Aurora-A) is a ubiquitous protein kinase regulating entry into mitosis and shown to promote transformation upon overexpression. In order to gain information on the structural features determining its substrate specificity, we assayed human recombinant AurA on a variety of phosphoacceptor peptide substrates including a series of properly modified derivatives of the Kemptide (ALRRASLGAA). The data presented here show that AurA is a basophilic Ser/Thr protein kinase recognizing the consensus R/K/N-R-X-S/T-B, where B denotes any hydrophobic residue with the exception of Pro. We show that the presence of a Pro at position $n+1$ fully abrogates phosphorylation of the peptide substrate. Although the consensus for AurA is reminiscent of that of PKA (protein kinase A), it significantly differs from the latter for a much more stringent dependence on the hydrophobic residue at $n+1$ and for its tolerance of residues other than Arg at position $n-3$. Based on the finding that the peptide ALKRASLGAA is not a substrate of PKA while still providing a sensitive assay of AurA activity, we suggest that this peptide may be used for differential screening of the two kinases. We have further validated the AurA consensus by generating a peptide (APSSRRTT^{288}LCGT) that comprises the main AurA autophosphorylation site and by showing that AurA phosphorylated this peptide exclusively at one site fulfilling its consensus (Thr^{288}). Moreover, we show that AurA could autophosphorylate at Thr^{288} through an intermolecular mechanism of reaction and that, *in vivo*, PKA was not involved with Thr^{288} phosphorylation. The evidence obtained in the present study provides a rational tool for predicting AurA sites in potential substrates of physiological significance.

Key words: Aurora-A, consensus sequence, peptide substrate, phosphorylation, protein kinase, site specificity.

INTRODUCTION

Aurora protein kinases were initially identified in yeast and *Drosophila* for their role in the control of chromosome segregation and cytokinesis through the regulation of microtubule activity [1]. Budding yeast mutants for Ipl1, the single form of the Aurora kinase present in this organism [2], are characterized by abnormal ploidy [3]. Aurora mutants in *Drosophila* typically display monopolar spindles that result from defective centrosome separation [4]. Of the three human homologues so far described, AurA (Aurora-A) and AurB were isolated in a screening for kinases overexpressed in colon carcinoma [5]. AurA is expressed at low-levels in most tissues but is particularly abundant in tumour cell lines [6,7] and accordingly, the gene coding for AurA maps to a region frequently amplified in human tumours and cancer cell lines [5,6,8,9]. AurA, which is localized at the centrosomes during interphase and at the spindle throughout mitosis [10], displays a peak of kinase activity at G_2 phase (growth 2 phase), before CDC2 (cell division cycle 2 kinase) and AurB [5]. AurA kinase activity results from the balance of positive and negative phosphorylation: whereas autophosphorylation [11] and phosphorylation by as yet unknown upstream kinases [12] contribute to confer full AurA activation, phosphorylation by GSK3 (glycogen synthase kinase 3) at two sites close to the T-loop apparently triggers AurA autophosphorylation at an inhibitory site [13]. A further layer of complexity in the control of AurA activity is conferred by interaction with protein phosphatase 1, which results in mutual regulation of the two enzymatic activities [14]. Degradation of AurA occurs at mitotic exit and is mediated by the anaphase-promoting complex/cyclosome (APC/C) [15–17]. Depletion of AurA by siRNA (small interfering RNA) interference resulted in impaired activation of cyclin B/CDK1 (cyclin-dependent kinase 1) and was followed by an almost complete block of entry into mitosis [18]. On the other hand, overexpression of AurA was shown to cause transformation of Rat1 and NIH3T3 cells, which in turn could grow as tumours in nude mice [5]. Among the physiological targets of AurA so far identified are histone H3, BRCA1 (breast-cancer susceptibility gene 1) and p53 [19–21]. Although in all cases phosphorylation by AurA was shown to affect the substrate's biological properties, the sites phosphorylated in these proteins could not be demonstrated to be exclusive targets of AurA.

To gain information on physiologically relevant AurA targets, we set out to define the consensus sequence of this kinase. Using human recombinant AurA, expressed in *Escherichia coli* and purified to near homogeneity, we screened a set of synthetic peptides derived from the Kemptide as well as peptides modelled on other phosphoacceptor sites. This has led to the definition of the consensus sequence recognized by AurA and the identification of a number of local features acting as positive or negative specificity determinants.

MATERIALS AND METHODS

AurA cloning, expression and purification

Full-length human AurA was obtained by means of PCR using *Pfu* polymerase (Stratagene, Cambridge, U.K.). The first-strand

Abbreviations used: AurA, Aurora-A; CDC2, cell division cycle 2 kinase; CK1, casein kinase 1; DTT, dithiothreitol; HEK-293T cells, human embryonic kidney 293T cells; kd-AurA, kinase-dead AurA; PKA, protein kinase A; wt-AurA, wild-type AurA.

[1] Correspondence may be addressed to either of the authors (email sferrari@imcr.unizh.ch and lorenzo.pinna@unipd.it).

cDNA template was synthesized on polyadenylated mRNA purified from mitotic HeLa cells using M-MLV reverse transcriptase (Promega, Chilworth, Southampton, U.K.). The gene-specific forward and reverse primers used in the PCR were: 5′-CGCGGATCCATGGACCGATCTAAAGAAAACTGCATTTC-3′ and 5′-GGCGAGCTCCTAAGACTGTTTGCTAGCTGATTCTTTG-3′ respectively. The 1.2 kb PCR product was purified using the QIAquick PCR purification kit (Qiagen, Chatsworth, CA, U.S.A.), subcloned into pBluescript SK$^+$ (Stratagene) via BamHI/XhoI sites and controlled by sequencing. The wt (wild-type) and kd (kinase-dead) (Asp^{274} > Asn) AurA open reading frames were subcloned in pTXB3 (New England Biolabs, Hitchin, Herts., U.K.) and recombinant AurA was expressed as Intein–AurA fusion protein in the BL21 *E. coli* strain. In order to purify AurA, cells were lysed in prechilled buffer A (20 mM Tris, pH 8.0, 500 mM NaCl, 0.1 % Triton X-100, 1 mM EDTA and 10 % glycerol, containing Roche complete protease inhibitor cocktail) and extracts were sonicated before centrifugation for 30 min at 38 724 *g* in SS-34 rotor (Sorvall Centrifuges, Kendro Laboratory Products, Bishop's Stortford, Herts., U.K.) at 4 °C. The soluble fraction was filtered and loaded on to a 10 ml chitin column (New England Biolabs), pre-equilibrated in buffer A at 4 °C. After washing with 200 ml of buffer A, flow was stopped and the column was left overnight in buffer A containing 100 mM DTT (dithiothreitol) to facilitate intein self-splicing. On the following day, proteins were step-eluted using buffer A and aliquots of each fraction were examined by SDS/PAGE.

Cell culture

HeLa and HEK-293T (human embryonic kidney-293T) cells were maintained in Dulbecco's modified Eagle's medium (OmniLab, Mettmenstetten, Switzerland) supplemented with 10 % (v/v) fetal calf serum (Life Technologies, Basel, Switzerland), penicillin (100 units/ml) and streptomycin (100 μg/ml) (complete medium). For synchronization experiments, cells were seeded at 1×10^6 in 10-cm-diameter dishes and treated after 24 h with 2 mM thymidine (SynGen, Cambridge, U.K.) for 16 h, released for 8 h in complete medium and thymidine (2 mM) was added for a second period (15 h). The extent of synchronization was controlled by flow cytometric analysis of DNA. Transient transfections were performed using FuGene6 (Roche, Lewes, East Sussex, U.K.). Treatment of cells with the PKA (protein kinase A) inhibitor H89 (Calbiochem, Bad Soden, Germany) or the PKA activators forskolin and 8Br-cAMP (Calbiochem) are described in the Figure legends.

Western blotting

A polyclonal serum to AurA (AurA-Pab36) was generated in two rabbits (Clonestar, Brno, Czech Republic) using full-length AurA. IgGs were purified by FPLC on a Protein A–Sepharose column (Amersham Biosciences). The antibody to AurA phospho-Thr^{288} and CDC2 phospho-Tyr^{15} were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.). The antibodies to CDC2 and histone H3 phospho-Ser^{10} were from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Western-blot analysis was performed after separation of proteins on polyacrylamide gels under denaturing conditions. Proteins were transferred to PVDF membrane (Amersham Biosciences), the membrane was probed with specific antibodies and bands were revealed with the ECL® system (Amersham Biosciences).

MS analysis

Aliquots corresponding to 10 μg of purified wt- and kd-AurA were precipitated by the addition of 125 μg/ml sodium deoxycholate and 10 % (w/v) trichloroacetic acid [22]. Proteins were dissolved in electrophoresis sample buffer (100 mM Tris/HCl, pH 6.8, 100 mM DTT, 2 %, w/v, SDS, 30 % glycerol and 0.2 % Bromophenol Blue) [23] and heated for 5 min at 95 °C. Samples were alkylated with idoacetamide (55 mM) for 45 min in the dark before subjecting them to SDS/PAGE. The gel was stained with Coomassie Blue and bands corresponding to wt- or kd-AurA were excised and digested with 1 μg of trypsin (Promega) in 50 mM ammonium bicarbonate (pH 8.0) at 37 °C for 16 h [24]. The resulting peptides were analysed by capillary liquid chromatography tandem MS (LC-MS/MS) using a Magic C18 100 μm $\times$ 10 cm HPLC column (Spectronex, Basel, Switzerland) connected on line to an ion-trap Finnigan DecaXP (ThermoFinnigan, San Jose, CA, U.S.A.). A linear gradient from 5 to 50 % B [0.1 % formic acid and 80 % (v/v) acetonitrile in water] in A (0.1 % formic acid and 2 % acetonitrile in water) in 60 min was delivered with a Rheos 2000 HPLC system (Flux, Basel, Switzerland) at 100 μl/min. A precolumn flow splitter reduced the flow to approx. 300 nl/min and the peptides were manually loaded with a 10 μl Hamilton syringe on a peptide cap-trap (Michrom BioResources, Auburn, CA, U.S.A.) mounted in the injection loop of the MS. The eluting peptides were ionized by electrospray ionization, detected and the peptide ions were automatically selected and fragmented by collision-induced dissociation (MS/MS) in the ion-trap. Individual MS/MS spectra were compared against the known protein sequence using TurboSequest software [25]. Phosphorylated peptides were sequenced more than once.

Peptide synthesis

Solvents, resin and coupling reagents for peptide synthesis were from Applied Biosystems (Foster City, CA, U.S.A.). All resins and protected amino acids were purchased from Novabiochem (Laufelfingen, Switzerland). HPLC grade solvents were obtained from Merck (Darmstadt, Germany). Synthetic peptides were prepared by solid-phase peptide synthesis using an automated peptide synthesizer (model 431-A, Applied Biosystems). The fluoren-9-ylmethoxycarbonyl (Fmoc) strategy was used throughout the peptide chain assembly [26]. Wang resin (loading of 0.96 mmol/g) was used as solid support to obtain 0.1 mmol of each peptide. A parallel synthesis of the peptide APSSRRTTLCGT was also performed on the amino PEGA resin (Novabiochem) using the same procedure described above in order to obtain a permanently anchored peptide to be employed for direct Edman sequencing analysis. Side-chain-protecting groups were *tert*-butyloxycarbonyl for Lys and Trp, *trytyl* for Gln, Asn and Cys; *tert*-butyl ester for Asp and Glu; *tert*-butyl ether for Ser, Tyr and Thr; 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulphonyl for Arg. Coupling was performed with a single reaction for 40–50 min by a 0.45 M solution in *N*,*N*-dimethylformamide (DMF) of 2-(1-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and *N*-hydroxybenzotriazole (HOBt) in the presence of *N*-ethyldi-isopropylamine (DIEA), following the manufacturer's instructions. Peptide cleavage was usually performed by reacting the peptidyl-resins with trifluoroacetic acid/water/thioanisole/ethane dithiol/phenol (10 ml/0.5 ml/0.5 ml/0.250 ml/750 mg) for 2–2.5 h. The peptides were precipitated with ice-cold ethyl ether and isolated by centrifugation. Pellets were washed several times with ether, dissolved in abundant water and freeze-dried. Purity was checked by analytical reversed-phase HPLC on a 5 μm C18 Symmetry300 column, 4.6 $\times$ 250 mm (Waters, Milford, MA, U.S.A.) using a linear gradient of 5–40 % acetonitrile in 0.1 % trifluoroacetic acid at 1 ml/min. The purity of the peptides was in the range of 85–95 %.

The peptide AcFNRTSLPWQGLKAATKKQKY was purified by a preparative reversed-phase HPLC column (Prep Nova-Pak HR C18, 250 mm × 10 cm, 6 μm bead size; Waters) at 12 ml/min using a linear gradient as described above. The molecular mass of the peptide was confirmed by MS with direct infusion on a Micromass ZMD-4000 mass spectrometer (Waters-Micromass, Milford, MA, U.S.A.).

Edman sequencing

The peptide APSSRRTTLCGT, which was covalently bound to the beads of the solid support employed during synthesis, was phosphorylated under conditions described below and exhaustively washed to eliminate excess ATP and other reagents. Subsequently, few beads bearing the radiolabelled peptide were loaded on a Procise HT 491 protein sequencer (Applied Biosystems). Modified cartridge chemistry cycle was used to isolate ATZ amino acids, according to the manufacturer's instructions. No-flask cycle or HPLC gradient cycles were loaded. At every cycle the removed ATZ amino acids were quantitatively transferred to an external fraction collector connected to the ATZ port, using 90 % (v/v) methanol and 10 % water as solvent (S1). The collected fractions (600 μl) were supplemented with 2 ml scintillation cocktail and counted for 1 min in a liquid-scintillation counter. The recovered/measured ^{33}P radioactivity at every cycle was plotted against the primary sequence of the peptide substrate.

Phosphorylation assay

Phosphorylation reactions were performed by incubating the phosphorylatable protein or peptide substrate in 25 μl of a medium containing 50 mM Tris/HCl (pH 8), 10 mM $MgCl_2$, 1 mM DTT, 50 μM [γ-^{33}P]ATP (specific radioactivity, 1–2 μCi/nmol) and 6 ng of recombinant protein kinase AurA for 10 min at 37 °C. PKA activity was similarly assayed by incubating peptide substrates in a solution containing 50 mM Tris/HCl (pH 7.5), 10 mM $MgCl_2$, 10^{-6} M cAMP and 10–20 ng of PKA holoenzyme. All activity assays were linear with respect to time and enzyme concentration. The *in vitro* reactions shown in Figure 1 were performed in the conditions described above with the exception that [γ-^{32}P]ATP (specific activity, 5–10 μCi/nmol) was used. The phosphate incorporated into protein substrates was evaluated by subjecting samples to SDS/PAGE, staining and autoradiography. The radiolabelled peptides were isolated and quantified by a procedure using phosphocellulose filters [27]. K_m values were calculated from Lineweaver–Burk double-reciprocal plots of the data. The values obtained represent the mean of at least three independent experiments.

Phosphoamino acid analysis

Aliquots of radiolabelled peptide substrates were subjected to partial acid hydrolysis in 6 M HCl at 105 °C for 4 h followed by high-voltage paper electrophoresis and corrections for hydrolytic loss of pSer (48 %) and pThr (14 %) as described previously [28]. The hydrolytic loss was not significantly influenced by the structure of the peptide substrates.

RESULTS

Purification and characterization of human recombinant AurA

BL-21 cells were transformed with plasmids encoding either wt- or kd-AurA as C-terminal fusion to the self-splicing protein intein. AurA was purified to near-homogeneity in one single chromatographic step (Figure 1A). Coomassie Blue stained bands corresponding to wt- and kd-AurA clearly displayed distinct electrophoretic mobility. Western-blot analysis performed with a purified AurA-Pab36 (Figure 1B), confirmed the identity of the protein bands observed in the Coomassie Blue-stained gel. Wt-AurA was capable of phosphorylating histone H3, whereas the kd-form was completely inactive (Figure 1C). The specific activity of the purified kinase was found to be 0.42×10^4 pmol · min^{-1} · mg^{-1} using histone H3 as substrate. Wt-AurA could also phosphorylate MBP (myelin basic protein) with comparable efficiency to histone H3, but was incapable of phosphorylating histone H1, a mixture of Lys-rich histones or PCNA (proliferating-cell nuclear antigen; Figure 1D).

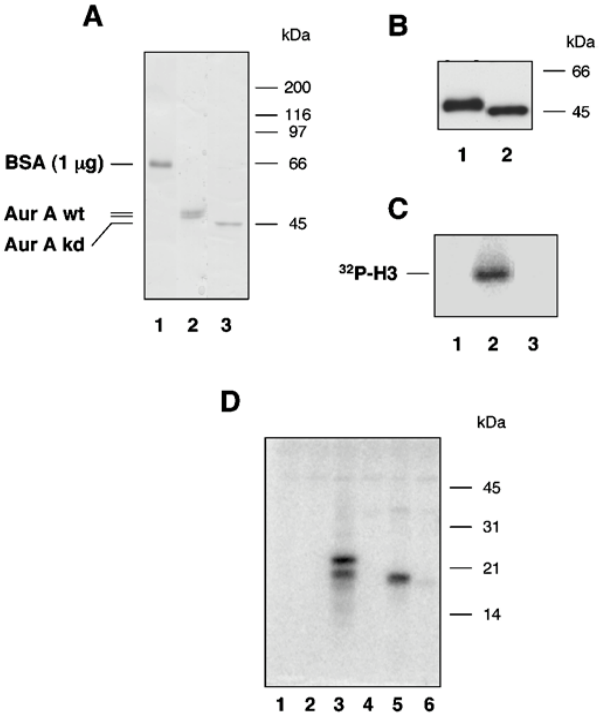


Figure 1 Analysis and characterization of recombinant AurA

(**A**) The extent of AurA purification was examined by resolving an aliquot of the first chitin-column fraction on an SDS/polyacrylamide gel. Aliquots derived from preparation of wt-AurA (lane 2) or kd-AurA (lane 3) are shown. To appreciate the protein amount, the Coomassie Blue signal given by 1 μg of BSA is shown in lane 1. (**B**) Aliquots of the same material were examined by Western-blot analysis using purified antibody AurA-Pab36. Lane 1, wt-AurA; and lane 2, kd-AurA. (**C**) Kinase activity of the purified fractions shown in (**A**) was assayed on histone H3, as indicated in the Materials and methods section. Lane 1, blank; lane 2, wt-AurA; and lane 3, kd-AurA. (**D**) Purified AurA was tested on a set of artificial substrates used at 0.1 mg/ml final concentration and resolved on 15 % (w/v) Laemmli gel. Lane 1, blank; lane 2, PCNA; lane 3, MBP; lane 4, histone H1; lane 5, histone H3; and lane 6, histone HIII-S (Lys-rich).

Specificity determinants of AurA

Preliminary screening of AurA activity was performed towards a wide spectrum of peptide substrates previously developed to characterize the consensus of a variety of protein kinases, including PKA, PKC, p90^{RSK}, CDC2, DYRK1α (dual-specificity tyrosine-phosphorylated and regulated kinase 1α), CK1 (casein kinase 1), CK2 and Golgi casein kinase [27]. The results obtained disclosed the basophilic nature of AurA, which proved inactive towards peptide substrates of acidophilic and proline-directed kinases (results not shown). Among the basic peptides readily phosphorylated by AurA the best one was a slightly extended

Table 1 Kinetic constants for AurA and PKA determined on a set of peptide substrates

Assays were performed in triplicate as indicated in the Materials and methods section; n.d., not determined due to undetectable phosphorylation. Residues whose displacement and/or substitution are discussed are shown in boldface.

		AurA			PKA		
	Substrate	K_m (μM)	K_{cat} (s^{-1})	Efficiency (K_{cat}/K_m) $\times 10^2$	K_m (μM)	K_{cat} (s^{-1})	Efficiency (K_{cat}/K_m) $\times 10^2$
1	AL**RR**ASLGAA	263	5.50	2.09	60	2.3	3.94
2	**ALA**RR**SLGAA**	200	0.39	0.19	1000	0.1	0.01
3	A**RR**AASLGAA	222	0.39	0.19	500	0.4	0.08
4	**RR**AAASLGAA	500	0.29	0.05	n.d.	n.d.	–
5	AL**KK**ASLGAA	n.d.	n.d.	–	n.d.	n.d.	–
6	AL**KR**ASLGAA	357	1.11	0.31	n.d.	n.d.	–
7	AL**RK**ASLGAA	1000	0.27	0.02	294	1.3	0.44
8	ALRRAS**I**GAA	370	5.36	1.45	44	2.1	4.77
9	ALRRAS**F**GAA	555	5.56	1.00	76	2.7	3.55
10	ALRRAS**R**GAA	1084	3.38	0.31	322	6.9	2.14
11	ALRRASt**BuGly**GAA	714	3.82	0.53	48	2.0	4.16
12	ALRRAS**A**GAA	430	0.32	0.07	53	1.3	2.45
13	ALRRAS**P**GAA	n.d.	n.d.	–	n.d.	n.d.	–
14	ALRRA**T**LGAA	5000	5.18	0.10	526	1.4	0.26
15	ALRRA**Y**LGAA	n.d.	n.d.	–	n.d.	n.d.	–
16	AcFNRT**S**LPWQGLKAATKKQKY	868	1.23	0.14	n.d.	n.d.	–
17	NKRRRSVTPPE	1010	1.91	0.18	41	1.22	2.97
18	NKRRRS**P**TPPE	n.d.	n.d.	–	n.d.	n.d.	–
19	Histone H3	10	0.35	3.50	n.d.	n.d.	–

version of the so-called Kemptide, ALRRASLGAA, a typical substrate of PKA as well as of several other basophilic protein kinases. This peptide was in fact phosphorylated by AurA with kinetic constants comparable with those of PKA (Table 1, peptide 1). Phosphorylation of Kemptide by PKA, as well as by a number of other basophilic protein kinases, is specified by the two arginine residues at positions $n-2$ and $n-3$. This was also the case of AurA, as shown by the detrimental effect of replacing both arginine residues with lysines (compare peptide 1 with peptide 5) and of transposing them to positions $n-4/n-5$ (peptide 4). Although both these modifications also abrogated phosphorylation by PKA, other substitutions disclosed significant differences between the sequence specificities of AurA and PKA. Of special interest in this respect is the effect of replacing the hydrophobic residue at position $n+1$ (Leu): substitution of this single residue with Ala (peptide 12) caused an approx. 30-fold reduction in phosphorylation efficiency by AurA. This was mostly accounted for by a significant decrease in K_{cat}, whereas its effect on PKA-catalysed phosphorylation was modest, entirely accounted for by just a 2-fold decrease in K_{cat}. This suggested that, in the case of AurA, a hydrophobic residue at position $n+1$ is a specificity determinant almost as powerful as the Arg-Arg doublet at positions $n-2/n-3$. To corroborate this conclusion, we introduced additional substitutions of Leu at $n+1$. The data showed that Ile and Phe, unlike Ala, were still compatible with high phosphorylation efficiency. However, whereas the $n+1$ substitution to Arg was relatively deleterious on both K_m and K_{cat} in the case of AurA, it almost tripled the K_{cat} with PKA. The latter was also insensitive to the replacement of the $n+1$ Leu with the artificial bulky apolar residue of *tert*-butyl-glycine, which in contrast hampered phosphorylation by AurA. It is worth noting that a Pro residue at position $n+1$ (peptide 13) completely abrogated phosphorylation by AurA as well as by PKA. The special relevance of a hydrophobic determinant at position $n+1$ in AurA peptide substrates may also explain why peptide 4, in which $n-2$ and $n-3$ are occupied by Ala while Leu is still present at $n+1$, was no more a substrate for PKA but was still significantly phosphorylated by AurA. The efficiency of phosphorylation of peptide 4 by AurA was comparable with that of peptide 12: in the latter, the Arg-Arg doublet was still at its optimal position but the Leu at $n+1$ was replaced by Ala.

Also remarkable was the different response of AurA and PKA to the individual substitution of the two crucial arginine residues with lysines: AurA tolerated much better the substitution at position $n-3$, the one at position $n-2$ being hardly compatible with detectable phosphorylation (compare peptides 6 and 7). The opposite applied to PKA, which still appreciably phosphorylated the Arg-Lys peptide while failing to phosphorylate the Lys-Arg peptide (peptide 6). Consequently, the latter may represent a promising lead for the development of specific substrates to selectively monitor AurA. In the same vein, it is worth remarking that AurA phosphorylated fairly efficiently a peptide encompassing the 213–232 sequence of CK1 (AcFNRT**S**LPWQGLKAATKKQKY) (peptide 16). This peptide, which was phosphorylated by AurA at a single Ser residue flanked by an Arg at $n-2$ and an Asn at $n-3$ (motif: NRxSL), was instead unaffected by PKA.

To sum up, it can be concluded that the consensus sequence of AurA conforms to the motif R/K/N-**R**-X-S/T-**B**, where B stands for any hydrophobic residue except Pro. The relevance of the hydrophobic in $n+1$ is comparable with that of the Arg at position $n-2$. Not surprisingly therefore, a peptide reproducing the segment around CDC25B Ser^{353}, recently identified as an AurA target [29], in which both crucial determinants are present (peptide 17 in Table 1) was readily phosphorylated by AurA with favourable kinetic constants. Interestingly, also in this case phosphorylation was abrogated if the Val at position $n+1$ is replaced by Pro (see peptides 17 and 18 in Table 1).

Identification of the residues phosphorylated in bacterially expressed AurA

Activation of AurA is associated with phosphorylation at the T-loop Thr^{288} [30]. In order to examine whether the human recombinant AurA that we expressed in *E. coli* was phosphorylated, we submitted wt- and kd-AurA to LC-MS/MS analysis. Among the approx. 100 tryptic peptides that were analysed,

Table 2 Identification of the sites of phosphorylation in AurA

Tryptic peptides derived from digestion of human recombinant wt-AurA were analysed by MS/MS. The sites of phosphorylation identified in phosphopeptides present among the peptides detected are indicated.

Sequence	Residues	Phosphorylated residue
SKENCISGPVK	4–14	Ser^{10}
SKENCISGPVKATAPVGGPK	4–23	Thr^{16}
RVLVTQQFPCQNPLPVNSGQAQR	24–46	Ser^{41}
VLCPSNSSQR	47–56	Ser^{53} or Ser^{54}
LVSSHKPVQNQK	64–75	Ser^{67}
QLQATSVPHPVSR	78–90	Ser^{83}
SKQPLPSAPENNPEEELASK	98–117	Ser^{98}
SKQPLPSAPENNPEEELASK	98–117	Ser^{104}
GKFGNVYLAR	142–151	Tyr^{148}
ELQKLSKFDEQR	221–232	Ser^{226}
DIKPENLLLGSAGELK	256–271	Ser^{266}
IADFGWSVHAPSSR	272–285	Ser^{278}
RTTLCGTLDYLPPEMIEGR	286–304	Thr^{287}
RTTLCGTLDYLPPEMIEGR	286–304	Thr^{288}
RISRVEFTFPDFVTEGAR	340–357	Ser^{342}
EVLEHPWITANSSKPSNCQNK	376–396	Ser^{391}

resulting in coverage of 70–80 % of the protein sequence, we could identify 14 phosphopeptides in wt-AurA, accounting for a total of 15 phosphorylation sites (Table 2). No phosphopeptide could be detected in kd-AurA, consistent with the view that all the phosphates were incorporated through an autocatalytic mechanism. Figure 2 shows the MS/MS spectra of two representative phosphopeptides with the assignment of the respective sites of phosphorylation. Somewhat surprisingly, only one among all sites identified (i.e. Thr ^{288} in the activation loop) fully conforms to the consensus outlined above, whereas another residue, Ser^{342}, partially matches it for displaying the K-R-X-S motif. Particularly striking was the identification of Tyr^{148} among the phosphorylation sites present in the recombinant kinase, which flatly contradicts the failure of AurA to phosphorylate its optimal peptide substrate with Ser replaced by Tyr (Table 1, peptide 15).

AurA specifically phosphorylated Thr^{288} within a synthetic peptide derived from its activation loop

MS analysis disclosed up to 15 residues phosphorylated in bacterially expressed AurA with only a couple of them displaying the motif R/K-R-X-S/T. This finding prompted us to synthesize peptides reproducing some of these sites to assess the significance of their phosphorylation. One such peptide encompassed the 281–292 sequence. This included the only phosphorylated residue (Thr^{288}) conforming to the optimal R-R-X-S/T-B consensus, along with another site found phosphorylated in recombinant AurA (Thr^{287}) and three potential phosphoacceptor residues. Two additional peptides were synthesized in order to reproduce the segments 36–45 and 78–87. These included the phosphorylated residues Ser^{41} and Ser^{83}, neither of which conformed to the AurA consensus, although the latter displayed the hydrophobic determinant at position $n+1$. As shown in Figure 3, only the 281–292 peptide was phosphorylated by AurA, whereas the other two peptides were totally unaffected. Upon phosphorylation by AurA, the 281–292 peptide was subjected to automated-Edman degradation to localize the phosphorylated residue(s). The data shown in Figure 4 provide the unambiguous demonstration that the only residue phosphorylated in this peptide was Thr^{288}, while no detectable amount of radiolabelled phosphate was released from Ser^{283}, Ser^{284}, Thr^{287} or Thr^{292}. The Thr^{288} homologue in *Xenopus* AurA (Thr^{295}) was found to be almost 100 % phosphorylated upon expression of the recombinant kinase in *E. coli* [11]. Interestingly, Thr^{288} is the structural homologue of PKA Thr^{197}, a crucial residue the phosphorylation of which is a prerequisite for the activation of PKA as well as a number of other 'Arg-Asp' protein kinases [31,32]. As for AurA (Figure 1A), autophosphorylation of bacterially expressed PKA at Thr^{197} was reported to cause retarded migration of the protein in SDS/PAGE [33]. To confirm our findings on the mechanism of AurA autophosphorylation, we examined the ability of active AurA to phosphorylate Thr^{288} in full-length kd-AurA *in vitro*. Incubation of catalytic amounts of wt-AurA with kd-AurA resulted in evident phosphorylation of the latter as shown by incorporation of ^{32}P (Figure 5B) as well as by reactivity to an antibody directed to phospho-Thr^{288} (Figure 5A).

In vivo phosphorylation of AurA at Thr^{288} occurs in a PKA-independent manner

Given the results described above, we set out to assess the claim that PKA might be responsible for AurA phosphorylation at Thr^{288} *in vivo* [30]. To this end, HeLa cells were synchronized by double-thymidine block-release and treated with nocodazole to prevent cells to move beyond mitosis. The degree of cell synchronization was controlled by flow cytometric analysis of DNA (Figure 5C) and phosphorylation of the mitotic marker histone H3 (Figure 5D). The PKA inhibitor H89 or either one of the two PKA activators forskolin and 8Br-cAMP was added at 8 h post-release and cells were analysed at 10 h. In synchronized cells, AurA protein level resulted to be low at G_1/S and high at G_2/M, as described in the literature [5]. Mitotic phosphorylation of AurA at Thr^{288} could be detected in control cells as well as in cells treated with the PKA inhibitor H89 (Figure 5D, lanes 2 and 3). The slightly lower level of Thr^{288} phosphorylation observed in cells that were treated with PKA activators (Figure 5D, lanes 4 and 5) was paralleled by a decrease rate of mitotic entry in this setting. This was visually estimated from the lower number of rounded-up cells ($\sim$30 % in treated versus $\sim$70 % in control cells) and by the lower extent of histone H3 phosphorylation in cells treated with PKA activators (Figure 5D, lanes 4 and 5). To extend this observation we employed HEK-293T cells, which are amenable to transient expression studies and display a slightly shorter cell cycle than HeLa. Double-thymidine synchronized HEK-293T cells were transfected with kd-AurA between the two blocks and treated with nocodazole after release from the second block. H89, forskolin or 8Br-cAMP was added 5 h post-release and cells were examined at 8 h. The extent of Thr^{288} phosphorylation in Myc-tagged kd-AurA appeared to increase with progression to mitosis (Figure 5E). This was not surprising, as it probably resulted from an intermolecular autophosphorylation reaction catalysed by endogenous AurA, similar to the one observed *in vitro* (Figures 5A and 5B). As for HeLa, HEK-293T cells showed no change in the pattern of Thr^{288} phosphorylation upon treatment with the PKA inhibitor H89 (Figure 5E, lane 3). The PKA activators forskolin or 8Br-cAMP resulted in a pattern of delayed entry into mitosis, although less pronounced compared with HeLa cells, and a proportional decrease of Thr^{288} phosphorylation (Figure 5E, lanes 4 and 5).

DISCUSSION

Mitosis is a highly regulated process during which sister chromatids are segregated into two newly formed cells [34]. Errors in this process may lead to aneuploidy and facilitate the

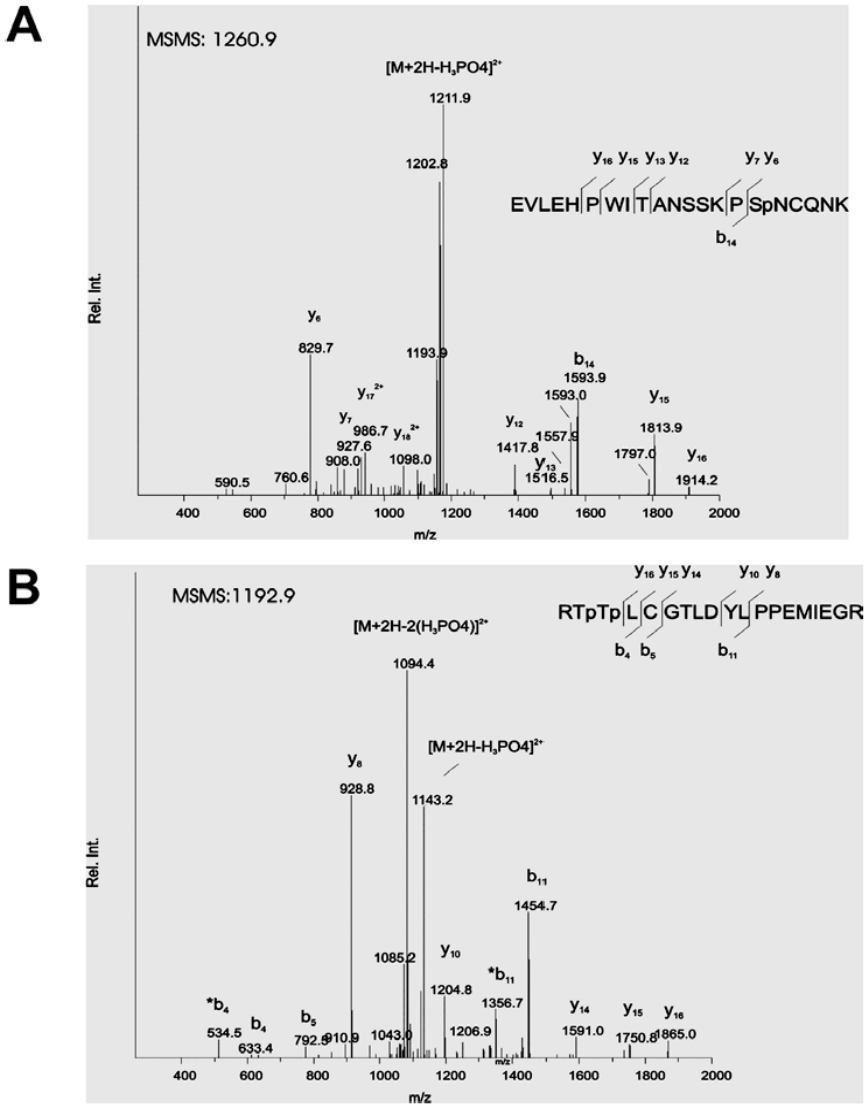


Figure 2 MS/MS spectra of two tryptic phosphopeptides identified by TurboSequest

(**A**) MS/MS spectrum of *m/z* 1260.9. The major fragment generated by neutral loss of H_3PO_4, indicative of phosphorylation, is marked $[M + 2H\text{-}H_3PO_4]^{2+}$. The y- (▾) and b-fragments (▸) detected upon collision-induced fragmentation are indicated in the sequence. The ions y_6, y_7 and b_{14} show that phosphorylation is located in the C-terminal part of the peptide allowing assignment of phosphorylation to the indicated Ser (Sp). (**B**) MS/MS spectrum of *m/z* 1192.9. The neutral losses of $1 \times H_3PO_4$ and $2 \times H_3PO_4$, marked as $[M + 2H - H_3PO_4]^{2+}$ and $[M + 2H - 2(H_3PO_4)]^{2+}$ respectively indicate two phosphorylation sites in this peptide. The ions y_{14}–y_{16} and b_4 and b_5 allowed location of the sites of phosphorylation to the N-terminus of the peptide, with the two indicated Thr (Tp) assigned as phosphorylation sites.

onset of cancer [35]. Central to the correct execution of mitosis is the protein kinase CDC2, although a number of studies have pointed to the essential auxiliary role played by Polo-like, NIMA (never in mitosis in *Aspergillus nidulans*)-related and Aurora-related kinases [34,36]. Despite the growing evidence on the importance of AurA in mitosis, clarification of its exact role requires the identification of physiological substrates and, in turn, of downstream pathways triggered by the kinase. One way to address this issue is to define the specificity determinants for recognition and phosphorylation of substrates by the kinase. In the present study, we have taken such an approach by testing human recombinant AurA on a set of peptide substrates appropriately designed to elucidate its site specificity.

A library of peptide substrates previously developed to assay a wide variety of protein kinases was used for a preliminary inspection of the site specificity of AurA. Once ascertained that only basic peptides were appreciably phosphorylated by AurA, we set out to precisely define the local structural determinants recognized by AurA. To this end, we synthesized a number of derivatives of an extended version of the 'Kemptide' (ALRRRASLGAA), which was taken as representative of the best substrate for AurA. From this analysis, it appeared that an Arg at

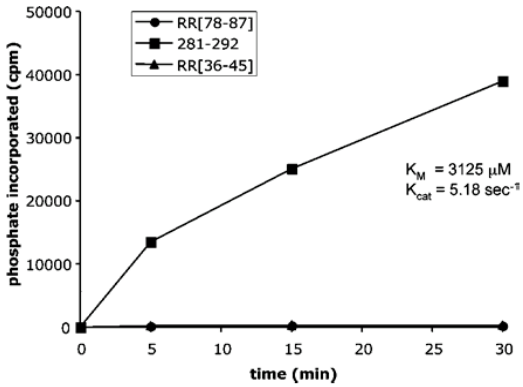


Figure 3 Phosphorylation of peptides reproducing AurA autophosphorylation sites

Synthetic peptides spanning residues 36–45, 78–87 and 281–292 of human AurA were subjected to *in vitro* phosphorylation with purified recombinant AurA. The K_m and K_{cat} values calculated for the optimal peptide substrate are indicated.

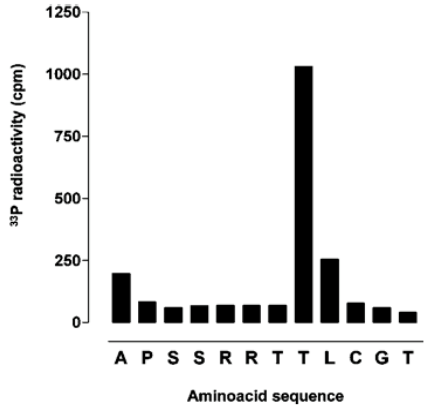


Figure 4 Identification of the phosphoacceptor residue within the peptide 281–292 of AurA

The synthetic peptide spanning residues 281–292 was subjected to *in vitro* phosphorylation with purified recombinant AurA followed by purification of the peptide and automated Edman degradation. Release of phosphate at each cycle of Edman degradation was quantified by liquid scintillation. The signal obtained at cycle 1 was probably due to the fact that phosphorylation was performed using the peptide substrate covalently bound to the beads of the solid support employed during synthesis (see the Materials and methods section). Release of non-specifically bound phosphate may have occurred at the first cycle of Edman degradation.

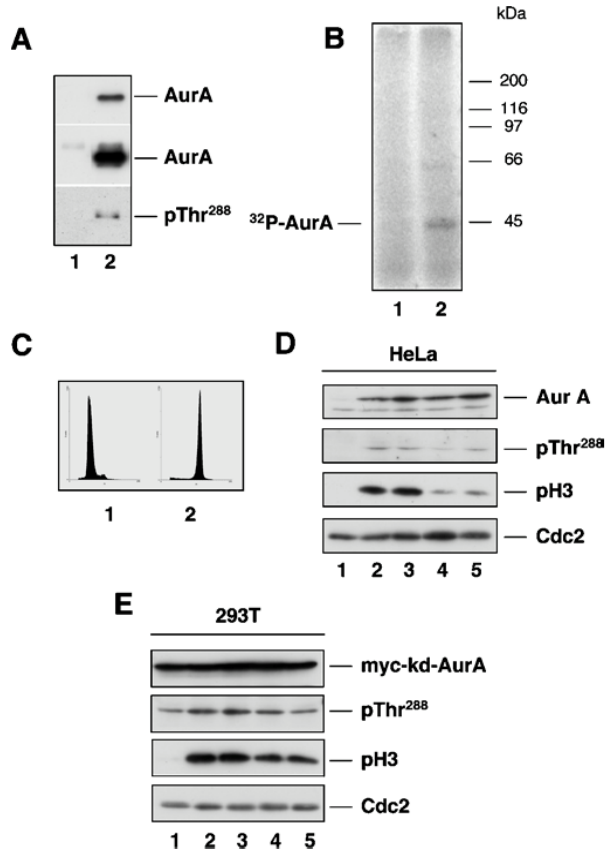


Figure 5 Effect of PKA inhibitors or activators on the *in vivo* phosphorylation of AurA Thr^{288}

In vitro phosphorylation reactions were performed using human recombinant wt-AurA (24 ng) incubated alone (lane 1) or in the presence of kd-AurA (750 ng, lane 2) under conditions described in the Materials and methods section. Reactions were performed using non-labelled ATP (**A**) or [γ-^{32}P]ATP (**B**). Aliquots of the reactions were resolved by SDS/PAGE and bands were either directly detected by autoradiography (**B**) or by Western-blot analysis using antibodies specific to AurA and to phosphorylated Thr^{288} (**A**). The upper and middle insets in (**A**) are different exposures of the same PVDF membrane that allow to appreciate the ratio of wt- and kd-AurA employed in the assay. (**C**) Flow cytometric analysis of double-thymidine synchronized (G_1/S, panel 1) and nocodazole-treated (G_2/M, panel 2) HeLa cells. (**D**) G_1/S synchronized HeLa cells (lane 1) were treated with nocodazole (0.4 ng/ml) 1 h after release from the second thymidine block (lanes 2–5). H89 (0.5 μM, lane 3), forskolin (20 μM, lane 4) or 8Br-cAMP (1 mM, lane 5) was added at 6 h and cells were harvested at 10 h post-release. AurA expression and phosphorylation at Thr^{288} as well as histone H3 phosphorylation were detected using antibodies described in the Materials and methods section. CDC2 was used as loading control. (**E**) G_1/S synchronized HEK-293T cells (lane 1) transfected with Myc-tagged kd-AurA were treated with nocodazole (0.4 ng/ml) 1 h after release (lanes 2–5). H89 (0.5 μM, lane 3), forskolin (20 μM, lane 4) or 8Br-cAMP were added at 5 h, cells were harvested at 8 h post-release and analysed as described in (**D**).

position $n-2$ and a hydrophobic residue at position $n+1$, relative to the phosphorylatable residue, are of crucial importance to determine site recognition by AurA. The resulting consensus sequence was R/K/N-R-X-S/T-B, where B stands for any hydrophobic residue, with the notable exception of Pro (see below). The sequence recognized by human AurA appeared to be similar to the putative consensus reported for yeast Ipl1, which was deduced on the basis of the sequence flanking sites phosphorylated by Ipl1 in a set of kinetochore proteins [37]. Although mammalian AurB is believed to be the functional homologue of yeast Ipl1, it is likely that, given the high conservation of the catalytic domains, the specificity of Aurora family members be similar. In the present study, we found that AurA shared with PKA, as well as with many other basophilic Ser/Thr protein kinases, the absolute requirement for an Arg at position $n-2$. However, whereas AurA displayed a stringent requirement for a hydrophobic residue at $n+1$, this seemed to be much less important for PKA. Additionally, AurA showed a wider tolerance of the nature of the $n-3$ residue, with a Lys or even an Asn, albeit in a peptide unrelated to the Kemptide (peptide 16, Table 1), fulfilling the criteria for phosphorylation. In contrast, PKA showed a stringent requirement for Arg at $n-3$. The inspection of PKA and AurA structures may provide

Table 3 Residues of AurA corresponding to the amino acids implicated in $p+1$, $p-2$ and $p-3$ recognition sites of PKA [38]

Recognition site	AurA	PKA
$p+1$	Leu^{289}	Leu^{198}
	Leu^{293}	Pro^{202}
	Leu^{296}	Leu^{205}
$p-2$	Glu^{260}	Glu^{170}
	Asp^{294}	Glu^{203}
	Glu^{321}	Glu^{230}
$p-3$	Thr^{217}	Glu^{127}
	–	Asp^{329}
	–	Glu^{331}

a partial explanation for their different site specificity. Whereas in fact all three acidic residues interacting with the Arg $n-2$ in the PKA/pseudosubstrate peptide complex [38] are conserved in AurA (Table 3), the corresponding three acidic residues in PKA that recognize the $n-3$ Arg are either absent from AurA, due to its shorter C-terminal domain, or replaced by a Thr (Thr^{217} instead of Glu^{127}). Moreover, the hydrophobic pocket of the $p+1$ loop of PKA, which is formed by Leu^{198}, Pro^{202} and Leu^{205} and functions by accommodating the side chain of the $n+1$ residue of the substrate, is conserved but more hydrophobic in AurA, with Pro^{202} replaced by a third Leu. This may account for the more stringent requirement of AurA for a hydrophobic determinant at $n+1$. Apart from the rationale underlying the different consensus of AurA and PKA, our results indicated that the peculiar requirement of AurA could be exploited for the development of specific peptide substrates to be used for monitoring AurA kinase activity. Such peptides will be unaffected or marginally affected by PKA and possibly other basophilic protein kinases. Two representative examples are peptides 6 and 16 of Table 1: neither of them was phosphorylated by PKA, while they proved to be fairly good substrates for AurA by virtue of their K/N-R-X-S-L motif. The performance of these peptides could be further improved by modifications aimed at optimizing their phosphoacceptor properties without loosing specificity. Useful hints may come from screening a peptide library where the Z-K-R-X-S-L-Z′ scaffold is maintained invariant while the X, Z and Z′ residues are randomly modified.

The AurA consensus, which was deduced from the analysis of Kemptide derivatives, was further validated by a parallel approach based on peptides encompassing sites that we found phosphorylated in the bacterially expressed kinase. MS analysis showed that the 15 sites present in human recombinant AurA (see Table 2) largely coincided with the 10 phosphorylated residues identified in the *Xenopus* kinase [11]. Especially relevant was Thr^{288}, the homologue of Thr^{197} in PKA. In both kinases the latter is located in the activation loop, also defined as the T-loop after PKA Thr^{197}. Phosphorylation of a residue in the activation loop has been reported to occur in a number of kinases and was shown to cause conformational changes that facilitate enzymatic activation [32]. A mechanistic explanation of the role of T-loop phosphorylation was provided by structural studies on the so-called 'Arg-Asp' kinases, in which an Arg adjacent to the catalytic Asp is believed to hamper catalysis unless it is neutralized by interaction with the phosphorylated residue of the activation loop [39]. AurA also contains an 'Arg-Asp' motif (residues 255–256) in the catalytic loop and the T-loop Thr^{288} is extensively phosphorylated in the bacterially expressed protein (the present study and [11]). This is consistent with an autocatalytic reaction and it has a rational explanation in the AurA consensus defined in the present study. The definite demonstration that Thr^{288} phosphorylation by AurA is driven by its consensus was provided by analysis of a peptide encompassing the 281–292 segment of AurA activation loop. This peptide was readily phosphorylated by AurA (Figure 3), but, more importantly, phosphorylation occurred at the only site (Thr^{288}) fulfilling the consensus for AurA among five potential phosphoacceptor residues present in the peptide (Figure 4). The ability of AurA to autophosphorylate at Thr^{288} was further confirmed using full-length kd-AurA as substrate for catalytically active AurA (Figures 5A and 5B). The finding that AurA could efficiently autophosphorylate at Thr^{288} questions the claim that PKA might be responsible for the phosphorylation of this site [30], particularly in view of the fact that cAMP level and, in turn, PKA activity decrease at the onset of mitosis [40]. Our results showing that *in vivo* phosphorylation at Thr^{288} was not affected by the PKA inhibitor H89 or the PKA activators forskolin and 8Br-cAMP, confirmed the doubts on the involvement of PKA in the process of AurA activation.

Another site displaying AurA consensus is Ser^{342}. Phosphorylation at Ser^{349} in *Xenopus* AurA (Ser^{342} in the human protein) was reported to be an autocatalytic event, which was apparently triggered by GSK3-mediated phosphorylation of two serine residues located at $n-4$ with respect to the T-loop residues Thr^{290} and Thr^{291} (Thr^{287} and Thr^{288} in human AurA). Autophosphorylation at Ser^{349} was claimed to result in the inhibition of *Xenopus* AurA activity [13]. Nonetheless, both human (the present study) and *Xenopus* AurA [11] were found to be fully phosphorylated at Ser^{342} and Ser^{349} respectively and, despite this, to be highly active kinases. It is also interesting to note that Thr^{287} and several other residues that do not fulfil the consensus for AurA were found to be phosphorylated in bacterially expressed AurA, albeit to an extent lower than what was observed for Thr^{288} ([11] and the present study, results not shown). Phosphorylation at these sites was probably an artifact due to the conditions of bacterial expression, where protein kinases reach abnormally high concentrations that are never observed under physiological conditions. This, in turn, could favour the reversible formation of aggregates where misphosphorylation of exposed residues would occur irrespective of their location in consensus sequences. A situation similar to what we observed with AurA has been reported for other protein kinases. Notably, bacterial expression of the recombinant catalytic subunit of PKA resulted in phosphorylation at Thr^{197} and at several other residues that, contrary to Thr^{197}, do not conform to the PKA consensus sequence [33]. The fact that some of the phosphorylation sites identified in bacterially expressed AurA may be the result of unspecific events is also supported by two observations: first, synthetic peptides reproducing two of the sites found in the bacterially expressed kinase and lacking consensus for AurA proved totally refractory to phosphorylation by AurA (Figure 3); secondly, one of the phosphosites identified in both human (the present study) and *Xenopus* AurA [11] was Tyr^{148}. Nonetheless, replacement of Ser with Tyr in the synthetic peptide displaying the optimal consensus for AurA resulted in undetectable phosphorylation of this peptide (Table 1).

Another interesting point emerging from our screening is the importance of the nature of the $n+1$ residue in the peptide substrate: a Pro at this position entirely abrogated phosphorylation by AurA. Such an absolute lack of recognition of the S/T-P motif, which is common to other Ser/Thr protein kinases [41], may reflect a device that is in place in the cell to control the onset of mitosis: by allowing synchronization of activity without overlapping of function with proline-directed kinases, AurA and CDC2 can regulate independent processes during the progression to and the execution of mitosis. Incidentally, the intolerance for a

Pro at $n+1$, in conjunction with lack of any residual consensus for AurA around Ser^{315} in p53 (TSSSS^{315}PQP), would argue against the possibility that this site might be efficiently phosphorylated by AurA. A synthetic peptide including Ser^{315} (residues 309–321) was entirely unaffected by AurA *in vitro* (results not shown). On the other hand, the finding that Ser^{315} is phosphorylated in full-length p53, although less readily than Ser^{215} [42], supports the view that sometimes higher-order structural features may enable AurA to phosphorylate residues that do not display the consensus sequence. In sharp contrast, Ser^{353} in CDC25B, which is embedded in the sequence RRRS^{353}V and which was recently reported to be a target of AurA [29], perfectly fits in with the consensus outlined with our peptide substrates.

It should be borne in mind in this connection that, although the substrate's primary sequence around target sites is of key importance to determine phosphorylation, this is likely to be one of the requirements for substrate recognition by protein kinases, which is also contributed by higher-order structures, supra-molecular association, compartmentalization etc. Pertinent to this remark is the case of histone H3, a putative target of AurA [19]: although histone H3 was phosphorylated much more slowly than several peptide substrates, it displayed a K_m value (10 μM) that was 26-fold lower than that of the optimal peptide substrate (Table 1). Despite this, however, the use of peptide substrates remains an excellent choice to determine the optimal consensus for protein kinases, and definition of such a consensus, in turn, facilitates the prediction of potential sites of phosphorylation in physiological substrates.

We are indebted to P. Sassone-Corsi, IGBMC (Institute of Genetics and Molecular and Cellular Biology), Strasbourg, France, for providing the AurA kinase-dead cDNA construct and to C. Koenig for invaluable technical assistance. This study was supported by the European grant LSHB-CT-2004-503467 and a grant from CIB (Consorzio Interuniversitario Biotecnologie) to L. A. P. and by a Zurich-Cancer-League grant and the Swiss National Science Foundation grant 31-100090/1 to S. F.

REFERENCES

1 Giet, R. and Prigent, C. (1999) Aurora/Ipl1p-related kinases, a new oncogenic family of mitotic serine-threonine kinases. J. Cell Sci. **112**, 3591–3601
2 Chan, C. S. and Botstein, D. (1993) Isolation and characterization of chromosome-gain and increase-in-ploidy mutants in yeast. Genetics **135**, 677–691
3 Biggins, S., Severin, F. F., Bhalla, N., Sassoon, I., Hyman, A. A. and Murray, A. W. (1999) The conserved protein kinase Ipl1 regulates microtubule binding to kinetochores in budding yeast. Genes Dev. **13**, 532–544
4 Glover, D. M., Leibowitz, M. H., McLean, D. A. and Parry, H. (1995) Mutations in aurora prevent centrosome separation leading to the formation of monopolar spindles. Cell (Cambridge, Mass.) **81**, 95–105
5 Bischoff, J. R., Anderson, L., Zhu, Y., Mossie, K., Ng, L., Souza, B., Schryver, B., Flanagan, P., Clairvoyant, F., Ginther, C. et al. (1998) A homologue of *Drosophila* aurora kinase is oncogenic and amplified in human colorectal cancers. EMBO J. **17**, 3052–3065
6 Zhou, H., Kuang, J., Zhong, L., Kuo, W. L., Gray, J. W., Sahin, A., Brinkley, B. R. and Sen, S. (1998) Tumour amplified kinase STK15/BTAK induces centrosome amplification, aneuploidy and transformation. Nat. Genet. **20**, 189–193
7 Bischoff, J. R. and Plowman, G. D. (1999) The Aurora/Ipl1p kinase family: regulators of chromosome segregation and cytokinesis. Trends Cell Biol. **9**, 454–459
8 Sen, S., Zhou, H. and White, R. A. (1997) A putative serine/threonine kinase encoding gene BTAK on chromosome 20q13 is amplified and overexpressed in human breast cancer cell lines. Oncogene **14**, 2195–2200
9 Shindo, M., Nakano, H., Kuroyanagi, H., Shirasawa, T., Mihara, M., Gilbert, D. J., Jenkins, N. A., Copeland, N. G., Yagita, H. and Okumura, K. (1998) cDNA cloning, expression, subcellular localization, and chromosomal assignment of mammalian aurora homologues, aurora-related kinase (ARK) 1 and 2. Biochem. Biophys. Res. Commun. **244**, 285–292
10 Stenoien, D. L., Sen, S., Mancini, M. A. and Brinkley, B. R. (2003) Dynamic association of a tumor amplified kinase, Aurora-A, with the centrosome and mitotic spindle. Cell Motil. Cytoskeleton **55**, 134–146
11 Haydon, C. E., Eyers, P. A., Aveline-Wolf, L. D., Resing, K. A., Maller, J. L. and Ahn, N. G. (2003) Identification of novel phosphorylation sites on *Xenopus laevis* Aurora A and analysis of phosphopeptide enrichment by immobilized metal-affinity chromatography. Mol. Cell. Proteom. **2**, 1055–1067
12 Littlepage, L. E., Wu, H., Andresson, T., Deanehan, J. K., Amundadottir, L. T. and Ruderman, J. V. (2002) Identification of phosphorylated residues that affect the activity of the mitotic kinase Aurora-A. Proc. Natl. Acad. Sci. U.S.A. **99**, 15440–15445
13 Sarkissian, M., Mendez, R. and Richter, J. D. (2004) Progesterone and insulin stimulation of CPEB-dependent polyadenylation is regulated by Aurora A and glycogen synthase kinase-3. Genes Dev. **18**, 48–61
14 Katayama, H., Zhou, H., Li, Q., Tatsuka, M. and Sen, S. (2001) Interaction and feedback regulation between STK15/BTAK/Aurora-A kinase and protein phosphatase 1 through mitotic cell division cycle. J. Biol. Chem. **276**, 46219–46224
15 Honda, K., Mihara, H., Kato, Y., Yamaguchi, A., Tanaka, H., Yasuda, H., Furukawa, K. and Urano, T. (2000) Degradation of human Aurora2 protein kinase by the anaphase-promoting complex–ubiquitin–proteasome pathway. Oncogene **19**, 2812–2819
16 Castro, A., Arlot-Bonnemains, Y., Vigneron, S., Labbe, J. C., Prigent, C. and Lorca, T. (2002) APC/Fizzy-related targets Aurora-A kinase for proteolysis. EMBO Rep. **3**, 457–462
17 Littlepage, L. E. and Ruderman, J. V. (2002) Identification of a new APC/C recognition domain, the A box, which is required for the Cdh1-dependent destruction of the kinase Aurora-A during mitotic exit. Genes Dev. **16**, 2274–2285
18 Hirota, T., Kunitoku, N., Sasayama, T., Marumoto, T., Zhang, D., Nitta, M., Hatakeyama, K. and Saya, H. (2003) Aurora-A and an interacting activator, the LIM protein Ajuba, are required for mitotic commitment in human cells. Cell (Cambridge, Mass.) **114**, 585–598
19 Crosio, C., Fimia, G. M., Loury, R., Kimura, M., Okano, Y., Zhou, H., Sen, S., Allis, C. D. and Sassone-Corsi, P. (2002) Mitotic phosphorylation of histone H3: spatio-temporal regulation by mammalian Aurora kinases. Mol. Cell. Biol. **22**, 874–885
20 Ouchi, M., Fujiuchi, N., Sasai, K., Katayama, H., Minamishima, Y. A., Ongusaha, P. P., Deng, C., Sen, S., Lee, S. W. and Ouchi, T. (2004) BRCA1 phosphorylation by Aurora-A in the regulation of G_2 to M transition. J. Biol. Chem. **279**, 19643–19648
21 Katayama, H., Sasai, K., Kawai, H., Yuan, Z. M., Bondaruk, J., Suzuki, F., Fujii, S., Arlinghaus, R. B., Czerniak, B. A. and Sen, S. (2004) Phosphorylation by aurora kinase A induces Mdm2-mediated destabilization and inhibition of p53. Nat. Genet. **36**, 55–62
22 Bensadoun, A. and Weinstein, D. (1976) Assay of proteins in the presence of interfering materials. Anal. Biochem. **70**, 241–250
23 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**, 680–685
24 Shevchenko, A., Wilm, M., Vorm, O. and Mann, M. (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal. Chem. **68**, 850–858
25 Gatlin, C. L., Eng, J. K., Cross, S. T., Detter, J. C. and Yates, III, J. R. (2000) Automated identification of amino acid sequence variations in proteins by HPLC/microspray tandem mass spectrometry. Anal. Chem. **72**, 757–763
26 Fields, G. B. and Noble, R. L. (1990) Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. Int. J. Pept. Protein Res. **35**, 161–214
27 Ruzzene, M. and Pinna, L. A. (1999) Assay of protein kinases and phosphatases using specific peptide substrates. In Protein Phosphorylation – A Practical Approach (Hardie, D. G., ed.), pp. 221–253, Oxford University Press, Oxford
28 Perich, J. W., Meggio, F., Reynolds, E. C., Marin, O. and Pinna, L. A. (1992) Role of phosphorylated aminoacyl residues in generating atypical consensus sequences which are recognized by casein kinase-2 but not by casein kinase-1. Biochemistry **31**, 5893–5897
29 Dutertre, S., Cazales, M., Quaranta, M., Froment, C., Trabut, V., Dozier, C., Mirey, G., Bouche, J. P., Theis-Febvre, N., Schmitt, E. et al. (2004) Phosphorylation of CDC25B by Aurora-A at the centrosome contributes to the G_2–M transition. J. Cell Sci. **117**, 2523–2531
30 Walter, A. O., Seghezzi, W., Korver, W., Sheung, J. and Lees, E. (2000) The mitotic serine/threonine kinase Aurora2/AIK is regulated by phosphorylation and degradation. Oncogene **19**, 4906–4916
31 Johnson, L. N., Noble, M. E. and Owen, D. J. (1996) Active and inactive protein kinases: structural basis for regulation. Cell (Cambridge, Mass.) **85**, 149–158
32 Nolen, B., Taylor, S. and Ghosh, G. (2004) Regulation of protein kinases: controlling activity through activation segment conformation. Mol. Cell **15**, 661–675
33 Yonemoto, W., Garrod, S. M., Bell, S. M. and Taylor, S. S. (1993) Identification of phosphorylation sites in the recombinant catalytic subunit of cAMP-dependent protein kinase. J. Biol. Chem. **268**, 18626–18632
34 Nigg, E. A. (2001) Mitotic kinases as regulators of cell division and its checkpoints. Nat. Rev. Mol. Cell Biol. **2**, 21–32
35 Gisselsson, D. (2003) Chromosome instability in cancer: how, when, and why? Adv. Cancer Res. **87**, 1–29

36 Carmena, M. and Earnshaw, W. C. (2003) The cellular geography of aurora kinases. Nat. Rev. Mol. Cell Biol. **4**, 842–854
37 Cheeseman, I. M., Anderson, S., Jwa, M., Green, E. M., Kang, J., Yates, III, J. R., Chan, C. S., Drubin, D. G. and Barnes, G. (2002) Phospho-regulation of kinetochore-microtubule attachments by the Aurora kinase Ipl1p. Cell (Cambridge, Mass.) **111**, 163–172
38 Knighton, D. R., Zheng, J. H., Ten Eyck, L. F., Xuong, N. H., Taylor, S. S. and Sowadski, J. M. (1991) Structure of a peptide inhibitor bound to the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. Science **253**, 414–420
39 Johnson, L. N. and Lewis, R. J. (2001) Structural basis for control by phosphorylation. Chem. Rev. **101**, 2209–2242
40 Grieco, D., Porcellini, A., Avvedimento, E. V. and Gottesman, M. E. (1996) Requirement for cAMP-PKA pathway activation by M phase-promoting factor in the transition from mitosis to interphase. Science **271**, 1718–1723
41 Pinna, L. A. and Ruzzene, M. (1996) How do protein kinases recognize their substrates? Biochim. Biophys. Acta **1314**, 191–225
42 Liu, Q., Kaneko, S., Yang, L., Feldman, R. I., Nicosia, S. V., Chen, J. and Cheng, J. Q. (2004) Aurora-A abrogation of p53 DNA binding and transactivation activity by phosphorylation of serine 215. J. Biol. Chem. **279**, 52175–52182

Received 25 February 2005/28 April 2005; accepted 4 May 2005
Published on the Internet 9 August 2005, doi:10.1042/BJ20050343

4. UNPUBLISHED DATA – ONGOING PROJECTS

1. LOOKING FOR AURORA A INTERACTION PARTNERS – p62

(project with collaboration with Dr Torsten Kleffmann and Functional Genomics Center, University of Zürich)

2. RNA INTERFERENCE IN CANCER CELL LINES OVEREXPRESSING AURORA A

(teamwork project with Christiane König)

1. LOOKING FOR AURORA A INTERACTION PARTNERS – p62

(project with collaboration with Dr Torsten Kleffmann and Functional Genomics Center, University of Zürich)

The goal of the study was to identify new interaction partners of Aurora A with potential distinction between normal and DNA damage conditions.

1.1. Introduction

Ubiquitin is a small polypeptide of 76 amino acids that can be covalently attached to other proteins through an isopeptide bond. Ubiquitination occurs through sequential steps catalyzed by activating (E1), conjugating (E2) and ligating (E3) enzymes. Monoubiquitination plays a role as an endocytosis signal and as a signal for polymerase switch in case of monoubiquitination of PCNA, whereas polyubiquitin chains target substrates for degradation by the proteasome. Using energy derived from ATP hydrolysis the 26S proteasome unfolds the substrate polypeptide chain and translocates it into an interior chamber where the substrate is hydrolyzed to produce small peptides. Ubiquitin is not degraded there since it is released from the substrate by deubiquitinating enzymes (Figure 2).

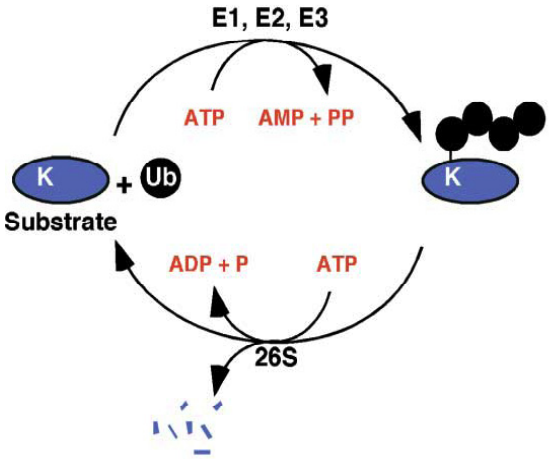


Figure 2. **Components and mechanisms in the ubiquitin/proteasome pathway** (from Pickart C.M., 2004), details in the text

E1 activates ubiquitin by using ATP to synthesize ubiquitin C-terminal adenylate, which then serves as an enzyme bound substrate for the formation of an E1- ubiquitin thiol ester.

The activated ubiquitin molecule is passed to a cysteine residue of the E2 component and from there, in an E3-dependent manner, to the substrate, where the isopeptide bond is formed between the activated C-terminal glycine of ubiquitin and an ε-amino group of a lysine in the substrate (Figure 3).

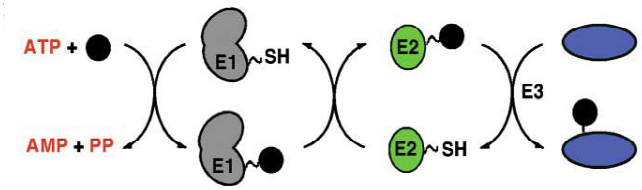


Figure 3. **The ubiquitin conjugation cascade.** (from Pickart C.M., 2004)

Following the linkage of the first ubiquitin, additional molecules of ubiquitin are attached to the previously conjugated moiety to form branched polyubiquitin chains employing lysine linkage K29, K48, or K63 (Pickart C.M., 2001). It has been postulated that the fate of a substrate depends on the length of the chain as well as the lysine linkage (K29, K48, or K63) involved in forming the chain. Proteins possessing K48 chains target proteins to the proteasome, whereas the ones with K63 have been shown to have other role than proteasome targeting. Ubiquitin itself is known to be modified at all seven lysine residues (K6, K11, K27, K33, K29, K48, and K63) adding additional potential diversity to polyubiquitin chains (Peng J., at al, 2003).

1.2. Materials and methods

1.2.1. Immunoprecipitation and mass spectroscopy

HeLa cells were treated with double thymidine block as described (Krystyniak A. et al, 2006). After 8h from the second thymidine release, cells were treated in the presence or absence of etoposide for subsequent 2 hours, then harvested. Immunoprecipitations of 10mg of total cell extract were carried out as described below, over night at 4^{o}C, with constant rotation, using 20 µl of anti-Aurora A polyclonal antibody and 50 µl of Protein A Sepharose beads (S. Cruz Biotech.). Immunoprecipitates were washed 3x 1 ml ice-cold

Buffer A (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 6 mM EGTA, 15 mM Na-pyrophosphate, 0.5 mM Na-orthovanadate, 1 mM benzamidine, 0.1 mM phenylmethylsulfonil fluoride (PMSF), 1% Nonidet P-40) and boiled (5 min at 95^0C) in SDS sample buffer, then loaded on the 10% polyacrylamide gel. The gel was stained with Coomasie Brilliant Blue to visualize protein bands. Each lane, corresponding to one immunoprecipitation was cut into 8 pieces and each piece was then prepared for MS separately. Gel pieces were digested with trypsin (Roche sequencing grade) according to a described protocol (Shevchenko A., et al, 1996). After overnight incubation the supernatant was removed and the remaining peptides were extracted three times with 80% acetonitrile / 10% formic acid. Samples were dried in a vacuum centrifuge and then subjected to MALDI-ToF-MS analysis.

The obtained peptide mass lists were searched against human protein data base ProteinPhophet.

1.2.2. Co-expression and co-immunoprecipitation

HEK-293T cells were maintained in DMEM (OmniLab) medium supplemented with 10% fetal calf serum (FCS, Life Technologies), penicillin (100U/ml) and streptomycin (100µl/ml).

Transfections, using myc-tagged Aurora A, flag-tagged p62 and empty pcDNA3 vector as mock control, were done using Metafectene (Biontex) according to the manufacturer's instructions. Cells were harvested for protein level analysis and IP experiment after 24h post transfection.

Cell extraction and detection of proteins by Western blot analysis was carried out as previously described (Charrasse *et al.*, 2000). Immunoprecipitations were carried out for 3h at 4^oC in Buffer A (described above) using 1mg of total protein. Proteins were immobilized on Protein A Sepharose beads (S. Cruz Biotech.) and washed in 3x 1 ml ice-cold Buffer A. 5 µl of polyclonal AurA-Pab36 antibody was used for each immunoprecipitation whereas detection by Western blot was performed with monoclonal antibody AurA-35C1 and anti-Flag antibody for p62.

1.3. Results

1.3.1. Mass spectroscopy

Combined and selected results of the mass spec analysis are presented in Table 1. Protein Q13446 was identified as Sequestosome-1/p62 (known also as: phosphotyrosine-independent ligand for the Lck SH2 domain of 62 kDa, ubiquitin-binding protein p62, EBI3-associated protein of 60 kDa, p60, EBIAP). The signal for p62 was found in both non-treated and eto-treated samples with protein probability (score) of 0.62 and 1.0, respectively. The sequence coverage was 5,9% in the sample of non-treated cells and 11,6% in the sample of cells treated with etoposide, which in the latter case corresponded to 3 different peptides.

1.3.2. Co-immunoprecipitation

In order to confirm the results from mass-spec analysis, showing that Aurora A interacts with p62, we co-transfect exponentially growing HEK-293T cells with 1μg (for 10 cm dish) for each of the two construct, namely myc-AurA and flag-p62. Western blot analysis confirmed the presence of both transfected proteins (lane 2 in Figure 5A and lane 3 in Figure 5B). The corresponding bands were not present in mock-transfected samples (lane 1 in 5A and lane 2 in 5B), confirming the specificity of the detection.
Immunoprecipitation was carried out using polyclonal AurA-Pab36 antibody as described in Materials and Methods. Western blot analysis of immunoprecipitated proteins using the anti-flag antibody detected a band of size corresponding to p62 (lane 4, Figure 5A). In order to further confirm the specificity of this interaction, the membrane was stripped and re-probed with monoclonal AurA-35C1 antibody (Figure 5A). The latter recognized ectopically expressed Aurora A both in total cell extract and in immunoprecipitated complexes. The lower band, visible only upon immunoprecipitation (lanes 4 and 5, Figure 5B) represents endogenous Aurora A. Due to its low level in asynchronously growing cells, this band is not detectable in Western blot analysis of total cell extracts.

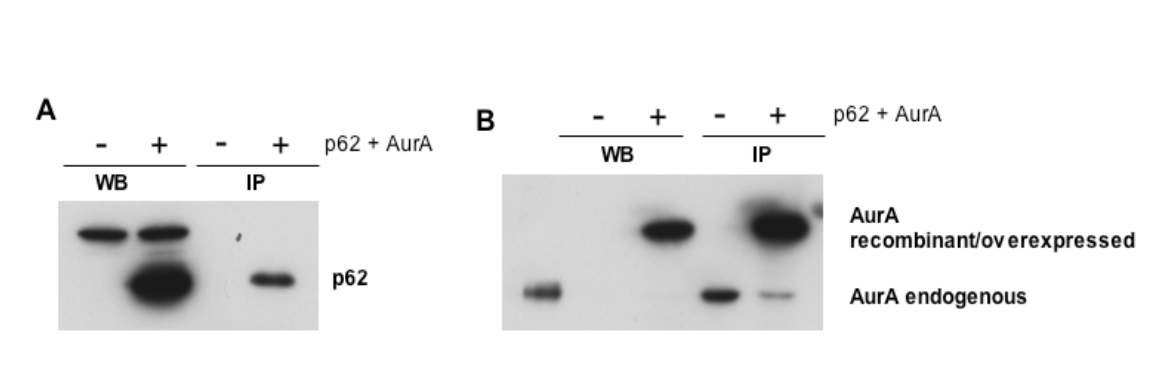


Figure 5. **Aurora A interacts with p62.** A. Detection of overexpressed p62 in WB (left part) and in co-immunoprecipitation (right panel). B. Detection of Aurora A (with monoclonal antibody) in WB (left part) and in immunoprecipitation (right part). First line is a recombinant purified kinase dead Aurora A, expressed and purified form *E.coli,* serving as a size and expression control.

1.4. Outlook

We were able to show the interaction between Aurora A and p62 by means of mass spectroscopy analysis and direct immunoprecipitation.

p62 is a cellular protein cloned as a co-interacting protein of the atypical protein kinase Cζ (aPKCζ) (Puls A. et al, 1997). p62 was shown to bind to the SH2 domain of $p56^{lck}$ in a phosphotyrosine-independent manner (Park I. et al, 1995). The ability of p62 to bind ubiquitin in a non-covalent fashion was mapped to amino acids 386–434 in the C-terminus of the protein, a region that shows homology with other proteins possessing an ubiquitin-associated (UBA) domain (Vadlamudi R.K., et al, 1996). Further studies have revealed that p62 has affinity for multi-Ub chains and may serve as a receptor to bind and store ubiquitinated proteins (Shin J. 1998). p62 contains several structural motifs, like acidic interaction domain (AID/ORCA/PC/PB1) that binds the aPKC, a ZZ finger, a binding site for the RING finger protein TRAF6, two PEST sequences, and, mentioned above, an UBA domain (scheme shown at Figure 1). The presence of these motifs suggests that p62 may participate in the formation of multimeric signal complexes.

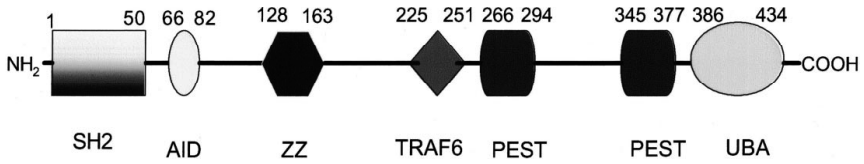


Figure 1. **A schematic diagram showing the domain organization of the p62 protein.** (after Geetha T. and Wooten M.W., 2002)

Recent studies confirmed that the UBA domain of p62 binds preferentially K63-polyubiqiutinated substrates and that it interacts with the proteasome (Seibenhener M.L., et al. 2004). It was shown that depletion of p62 results in inhibition of ubiquitin proteasome-mediated degradation and accumulation of ubiquitinated proteins, supporting the hypothesis of p62 as critical ubiquitin chain-targeting factor that shuttles substrates for proteasomal degradation.

Moreover, p62 is known to function as a scaffold in a range of signaling pathways associated with cell stress, survival and inflammation, and also controls transcriptional activation and protein recruitment to endosomes. The most well-described and extensively studied function of p62 is its role as a scaffold for selective activation of transcription factor NF-κB (Geetha T. and Wooten M.W., 2002).

Aurora A degradation is known to be dependent on the ubiquitin ligase activity of the APC/cyclosome, specifically on Cdh1. The detailed mechanism of regulation of this process remains to be explored. Our data, showing the interaction between Aurora A and p62, point to the possible involvement of p62 in the mechanism of shuttling poly-ubiquitinated Aurora A to the proteasome. Functional assays using siRNA technology will be now needed to support the relevance of Aurora A – p62 interaction at mitosis and to test the hypothesis on a possible role of p62 in the process of Aurora A degradation.

protein	probability	% coverage	num uniq peps	tot num peps	description NT	peptide sequence
IMB1_HUMAN	1	3.1	2	3	(Q14974) Importin beta-1 subunit (Karyopherin beta-1 subunit) (Nuclear factor P97) (Importin 90)	VLANPGNSQVAR AAVENLPTFLVELSR
LAMA_HUMAN, Q8N519, Q5TCJ4	1	7.1	3	4	(P02545) Lamin A\C (70 kDa lamin),(Q8N519) LMNA protein,(Q5TCJ4) Lamin A\\C	SGAQASSTPLSPTR EGDLIAAQAR TLDSVAKER
UBE1_HUMAN	1	7.2	5	5	(P22314) Ubiquitin-activating enzyme E1 (A1S9 protein)	AAVATFLQSVQVPEFTPK ALPAVQQNNLDEDLIR SLVASLAEPDFVVTDFAK DNPGVVTC[160]LDEAR LDQPMTEIVSR
STK6_HUMAN	1	39.5	11	22	(O14965) Serine\threonine-protein kinase 6 (EC 2.7.1.37) (Serine\threonine kinase 15) (Aurora\IPL1-related kinase 1) (Aurora-related kinase 1) (hARK1) (Aurora-A) (Breast-tumor-amplified kinase)	SKENC[160]ISGPVK SKQPLPSAPENNPEEELASK IADFGWSVHAPSSR FGNVYLAR EVEIQSHLR RVLVTQQFPC[160]QNPLPVNS GQAQR LSKFDEQR QLQATSVPHPVSRPLNNTQK LVSSHKPVQNQK VLC[160]PSNSSQRVPLQAQK ISRVEFTFPDFVTEGAR
YB1_HUMAN, Q7KZ24,Q15325	1	17.5	2	3	(P67809) Nuclease sensitive element binding protein 1 (Y box binding protein-1) (Y-box transcription factor) (YB-1) (CCAAT-binding transcription factor I subunit A) (CBF-A) (Enhancer factor I subunit A) (EFI-A) (DNA-binding protein B) (DBPB),(Q7KZ24) Nuclease sensitive element binding protein-1,(Q15325) Nuclease sensitive element binding protein 1	GAEAANVTGPGGVPVQGSK AADPPAENSSAPEAEQGGAE SVGDGETVEFDVVEGEK
H14_HUMAN, H12_HUMAN, H13_HUMAN, H11_HUMAN	1	11.3	2	2	(P10412) Histone H1.4 (Histone H1b),(P16403) Histone H1.2 (Histone H1d),(P16402) Histone H1.3 (Histone H1c),(Q02539) Histone H1.1	ALAAAGYDVEK GTGASGSFKLNKK
TADBP_HUMAN	0.99	4.3	1	1	(Q13148) TAR DNA-binding protein-43 (TDP-43)	FGGNPGGFGNQGGFGNSR

PRS7_HUMAN	0.98	3	1	2	(P35998) 26S protease regulatory subunit 7 (MSS1 protein)	FDDGAGGDNEVQR
DUS15_HUMAN	0.94	8.1	1	1	(Q9H1R2) Dual specificity protein phosphatase 15 (EC 3.1.3.48) (EC 3.1.3.16)	HRTSKTSGAQC[160]PPM[147]TSATC[160]LLAAR
Q6PJM1, Q9Y5B9	0.93	1.9	1	1	(Q6PJM1) SUPT16H protein (Fragment),(Q9Y5B9) Chromatin-specific transcription elongation factor FACT 140 kDa subunit	LTEQKGEQQIQK
G3B2_HUMAN	0.93	2.7	1	2	(Q9UN86) Ras-GTPase-activating protein binding protein 2 (GAP SH3-domain binding protein 2) (G3BP-2)	VEAKPEVQSQPPR
LAP4_HUMAN	0.91	1	1	1	(Q14160) LAP4 protein (Scribble homolog protein) (hScrib)	EIEEGQPEAPWTLPGGR
Q9BRB7, ACTY_HUMAN, ACTZ_HUMAN	0.88	2.7	1	1	(Q9BRB7) ARP1 actin-related protein 1 homolog B, centractin beta,(P42025) Beta-centractin (Actin-related protein 1B) (ARP1B),(P61163) Alpha-centractin (Centractin) (Centrosome-associated actin homolog) (Actin-RPV) (ARP1)	AGFAGDQIPK
ZSWM2_HUMAN	0.87	1.6	1	2	(Q8NEG5) Zinc finger SWIM domain containing protein 2	YSNCMGEITR
RPGF6_HUMAN	0.86	1.7	2	19	(Q8TEU7) Rap guanine nucleotide exchange factor 6 (PDZ domain containing guanine nucleotide exchange factor 2) (PDZ-GEF2) (RA-GEF-2)	SDNLSDSSHSEISSR HLQDLQDIFDPSR
LAM1_HUMAN	0.83	2.1	1	1	(P20700) Lamin B1	AGGPTTPLSPTR
MKRN1_HUMAN	0.76	2.5	1	1	(Q9UHC7) Makorin 1 (RING finger protein 61)	LILKYKEAM[147]SNK
RFA1_HUMAN	0.71	1.9	1	1	(P27694) Replication protein A 70 kDa DNA-binding subunit (RP-A)	SGGVGGSNTNWK

					(RF-A) (Replication factor-A protein 1) (Single-stranded DNA-binding protein)	
PA2G4_HUMAN	0.68	4.6	1	1	(Q9UQ80) Proliferation-associated protein 2G4 (Cell cycle protein p38-2G4 homolog) (hG4-1)	TAENATSGETLEENEAGD
Q13446	0.62	5.9	1	1	(Q13446) P60	AGEARPGPTAESASGPSEDPSVNFLK
Q7Z4K7,Q6ZTA4	0.56	2.1	1	1	(Q7Z4K7) TRIM9-like protein TNL (Tripartite motif-containing 67),(Q6ZTA4) Hypothetical protein FLJ44831	AQLSQALNGVSDKAK
ZN479_HUMAN	0.55	4	1	2	(Q96JC4) Zinc finger protein 479 (Zinc finger protein Kr19) (HKr19)	EKPYACEECGQAFSLSSNLM[147]R
PRKDC_HUMAN	0.53	0.2	1	2	(P78527) DNA-dependent protein kinase catalytic subunit (EC 2.7.1.37) (DNA-PKcs) (DNPK1) (p460)	LAC[160]DVDQVTR
KI21A_HUMAN	0.47	1.5	1	1	(Q7Z4S6) Kinesin family member 21A (Kinesin-like protein KIF2) (NY-REN-62 antigen)	KLSSSDAPAQDTGSSAAAVETDASR
Q7Z3P1, ZN449_HUMAN	0.46	2.8	1	2	(Q7Z3P1) Hypothetical protein DKFZp686G24119,(Q6P9G9) Zinc finger protein 449 (Zinc finger and SCAN domain containing protein 19)	C[160]THCSKSFR
MGMT_HUMAN	0.44	8.7	1	2	(P16455) Methylated-DNA--protein-cysteine methyltransferase (EC 2.1.1.63) (6-O-methylguanine-DNA methyltransferase) (MGMT) (O-6-methylguanine-DNA-alkyltransferase)	LGKPGLGGSSGLAGAWLK
BAZ2B_HUMAN, Q6MZK7	0.42	3.4	1	2	(Q9UIF8) Bromodomain adjacent to zinc finger domain 2B (hWALp4),(Q6MZK7) Hypothetical protein DKFZp686H10114	KC[160]NQEQSKNQPLDAR
PSB10_HUMAN	0.39	5.1	1	2	(P40306) Proteasome subunit beta type 10 precursor (EC 3.4.25.1)	ATNDSVVADKSC[160]EK

					(Proteasome MECl-1) (Macropain subunit MECl-1) (Multicatalytic endopeptidase complex subunit MECl-1)	
Q5T2B5, CUL2_HUMAN	0.36	1.1	1	1	(Q5T2B5) Cullin 2,(Q13617) Cullin homolog 2 (CUL-2)	CIEVLIDK
Q8IW93	0.26	1	1	1	(Q8IW93) Rho guanine nucleotide exchange factor (GEF) 19 (Novel protein)	SVEM[147]SGDR

protein	probability	% coverage	num uniq peps	tot num peps	description eto	peptide sequence
STK6_HUMAN	1	6.9	2	3	(O14965) Serine\threonine-protein kinase 6 (EC 2.7.1.37) (Serine\threonine kinase 15) (Aurora\IPL1-related kinase 1) (Aurora-related kinase 1) (hARK1) (Aurora-A) (Breast-tumor-amplified kinase)	SKQPLPSAPENNPEEELASK LSKFDEQR
IMB1_HUMAN	1	3.1	2	3	(Q14974) Importin beta-1 subunit (Karyopherin beta-1 subunit) (Nuclear factor P97) (Importin 90)	VLANPGNSQVAR AAVENLPTFLVELSR
Q13446	1	11.6	3	4	(Q13446) P60	AGEARPGPTAESASGPSEDPS VNFLK AYLLGKEDAAR LTPVSPESSSTEEK
LAP2A_HUMAN, LAP2B_HUMAN	0.99	8.6	2	2	(P42166) Lamina-associated polypeptide 2 isoform alpha (Thymopoietin isoform alpha) (TP alpha) (Thymopoietin-related peptide isoform alpha) (TPRP isoform alpha) [Contains: Thymopoietin (TP) (Splenin); Thymopentin (TP5)],(P42167) Lamina-associated polypeptide 2, isoforms beta\gamma (Thymopoietin, isoforms beta\gamma) (TP beta\gamma) (Thymopoietin-related peptide isoforms beta\gamma) (TPRP	GPPDFSSDEEREPTPVLGSGAA AAGR YGVNPGPIVGTTR

					isoforms beta\gamma) [Contains: Thymopoietin (TP) (Splenin); Thymopentin (TP5)]	
Q5TCJ4, LAMA_HUMAN,Q8N519	0.99	5.2	2	5	(Q5TCJ4) Lamin A\\C,(P02545) Lamin A\C (70 kDa lamin),(Q8N519) LMNA protein	SGAQASSTPLSPTR EGDLIAAQAR
Q5VW96, Q5VW97	0.99	3.2	1	1	(Q5VW96) Zinc finger protein 326,(Q5VW97) Zinc finger protein 326	ESVLTATSILNNPIVK
BCLF1_HUMAN	0.98	1.5	1	1	(Q9NYF8) Bcl-2-associated transcription factor 1 (Btf)	SSFYPDGGDQETAK
SSRP_HUMAN	0.97	3.4	1	2	(Q08945) Structure-specific recognition protein 1 (SSRP1) (Recombination signal sequence recognition protein) (T160) (Chromatin-specific transcription elongation factor 80 kDa subunit) (FACT 80 kDa subunit)	FYVPPTQEDGVDPVEAFAQNVLSK
RPGF6_HUMAN	0.7	0.9	1	11	(Q8TEU7) Rap guanine nucleotide exchange factor 6 (PDZ domain containing guanine nucleotide exchange factor 2) (PDZ-GEF2) (RA-GEF-2)	SDNLSDSSHSEISSR
Q8IW18, Q9H5L8, Q6PFW4, Q5XPI4	0.71	1.8	1	2	(Q8IW18) RNF123 protein (Fragment),(Q9H5L8) Hypothetical protein FLJ23315,(Q6PFW4) RNF123 protein (Fragment),(Q5XPI4) Ubiquitin ligase	RLAWVHATVYSQEK
MYCN_HUMAN	0.69	4.7	1	1	(P04198) N-myc proto-oncogene protein	GPPTAGSTAQSPGAGAASPAGR
Q6PCC6, ANC4_HUMAN	0.62	1.2	1	1	(Q6PCC6) Anaphase-promoting complex subunit 4,(Q9UJX5) Anaphase promoting complex subunit 4 (APC4) (Cyclosome subunit 4)	DTVGREGRDR
CECR1_HUMAN Q8NCJ2	0.61	9.6	1	1	(Q9NZK5) Cat eye syndrome critical region protein 1 precursor,(Q8NCJ2) Hypothetical protein	IKFPTVVAGFDLVGHEDTGHSLRDYK

					FLJ90221	
Q9Y266, Q9H0N2	0.54	2.7	1	1	(Q9Y266) MNUDC protein (SIG-92) (Nuclear distribution protein C homolog) (Nuclear distribution gene C homolog) (A. nidulans),(Q9H0N2) Hypothetical protein DKFZp566P0446	LSDLDSETR
ITCH_HUMAN	0.4	1.6	1	3	(Q96J02) Itchy homolog E3 ubiquitin protein ligase (EC 6.3.2.-) (Itch) (Atrophin-1-interacting protein 4) (AIP4) (NFE2-associated polypeptide 1) (NAPP1)	EFDPLGPLPPGWEK
DOCK4_HUMAN	0.27	0.5	1	1	(Q8N1I0) Dedicator of cytokinesis protein 4	TLISQCQTR
DUS16_HUMAN	0.27	2.3	1	1	(Q9BY84) Dual specificity protein phosphatase 16 (EC 3.1.3.48) (EC 3.1.3.16) (Mitogen-activated protein kinase phosphatase 7) (MAP kinase phosphatase 7) (MKP-7)	SCQMEFGESIM[147]SENR
Q96QP1	0.23	1.6	1	1	(Q96QP1) Lymphocyte alpha-kinase	TEIKNIDTVSTTQEKPHCQR
Q9H4A0, Q6DKQ9	0.2	0.8	1	1	(Q9H4A0) CDC2L5 protein kinase,(Q6DKQ9) Cell division cycle 2-like 5 (Cholinesterase-related cell division controller)	SPYSSRHSRSR
RFA1_HUMAN	0.21	1.8	1	1	(P27694) Replication protein A 70 kDa DNA-binding subunit (RP-A) (RF-A) (Replication factor-A protein 1) (Single-stranded DNA-binding protein)	LVM[147]SIRRSALM[147]
MINT_HUMAN	0.21	0.2	1	1	(Q96T58) Msx2-interacting protein (SPEN homolog) (SMART\HDAC1 associated repressor protein)	TPESAPENK

Table1. **Mass spectroscopy analysis of Aurora A interacting proteins.**

References:

Geetha T., Wooten M.W., "Structure and functional properties of the ubiquitin binding protein p62", 2002, *FEBS Letters*, 512: 19-24

Park I., Chung J., Walsh C.T., Yun Y., Strominger J.L., Shin J., "….", 1995, *Proc. Natl. Acad. Sci.,* USA, 92: 12338-12342

Peng J., Schwartz D., Elias J., Thoreen C., Cheng D., Marsischky G., Roelofs J., Finley D., Gygi S.P., "A proteomics approach to understanding protein ubiquitination", 2003, *Nat. Biotechnology,* 21: 921-926

Pickart C.M., "Mechanisms underlying ubiquitination", 2001, *Annu. Rev. Biochem.,* 70: 503-533

Pickart C.M., "Back to the future with ubiquitin", 2004, *Cell,* 116: 181-190

Puls A., Schmidt S., Grawe F., Stabel S., "Interaction of protein kinase C zeta with ZIP, a novel protein kinase C-binding protein", 1997, *Proc. Natl. Acad. Sci.,* USA, 94: 6191-6196

Seibenhener M.L., Babu J.R., Geetha T., Wong H.C., Krishna N., Wooten M.W., "Sequestosome 1/p62 is a polyubiquitin chain binding protein involved in ubiquitin proteasome degradation", 2004, *Molecular and Cellular Biology,* 24: 8055-8086

Shevchenko A., Wilm M., Vorm O., Mann M., "Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels", 1996, *Anal. Chem.,* 68: 850-858

Shin J., "P62 and the sequestosome, a novel mechanism for protein metabolism.", 1998, *Arch. Pharmacol. Res.,* 21: 629-633

Vadlamudi R.K., Joung I., Strominger J.L., Shin J., "p62, a phosphotyrosine-independent ligand of the SH2 domain of $p56^{lck}$, belongs to a new class of ubiqiutin binding proteins", 1996, *J. Biol. Chem.,* 271: 20235-20237

2. RNA INTERFERENCE IN CANCER CELL LINES OVEREXPRESSING AURORA A

(teamwork project with Christiane König)

2.1. Introduction and aim of study

The hallmark of cancers is genetic instability, which can be caused by specific gene mutations and/or acquired aneuploidy. One of the important players in maintaining genomic stability are centrosomes, the organelles establishing bipolar spindles during cell division and accurate segregation of chromosomes during cell division. Defect in centrosome number, organization and function may lead to chromosome amplification or loss and further to aneuploidy. Aurora A kinase plays an important role in chromosome segregation and centrosome functions. The gene encoding Aurora A is frequently amplified and overexpressed in various kinds of human cancer, including breast, colorectal, bladder, thyroid and others (Bischoff J.R., at al, 1998), (Royce M.E., et al, 2003), (Kamada K., et al, 2004), (Ulisse S., at al, 2006). Moreover, ectopic expression of Aurora A in mouse NIH3T3 cells and Rat1 fibroblasts causes centrosome amplification and transformation (Zhou H., et al, 1998). Those facts suggest that Aurora A may play a central role in the development of cancer.
We were able to show that treatment of cells with etoposide, an inhibitor of topoisomerase II that leads to the formation of double-strand breaks in DNA, caused inactivation of Aurora A, resulting in cell cycle arrest at the G2 phase. We could also show that such block was bypassed upon over-expression of wild type Aurora A (Krystyniak A., et el., 2006).

Drowning from the observations described above, we decided to test the hypothesis that elevated level of Aurora A in cancer cell lines may lead to higher resistance to etoposide. We postulated that in this case, down regulation of Aurora A by RNA interference would restore the sensitivity to etoposide. In a recently published study it was shown that down-regulation of Aurora A by siRNA in human pancreatic cancer cells enhanced the sensitivity to taxane and suppress tumor growth (Hata T., 2005).

2.2. Materials and methods

2.2.1. Cell lines, media and reagents

The human cervix carcinoma cell line HeLa and the human colorectal adenocarcinoma cell line CACO were maintained in DMEM (OmniLab) medium supplemented with 10% fetal calf serum (FCS, Life Technologies), penicillin (100U/ml) and streptomycin (100µl/ml). The human rectum adenocarcinoma cell line SW837 was maintained in the same medium with the additional 1% L-Glutamine. The human hereditary spherocytosis cell lines MT1 and TK6 were maintained in RPMI medium (Gibco) supplemented with 10% FCS and antibiotics. In order to perform shRNA experiments (see below) cell lines were tested for sensitivity to puromycin.
In order to obtain M-phase synchronization, cells were treated with Nocodazole (200ng/ml) for 15 hours.

2.2.2. MTT assay

Cells were seeded in 96-well plates in the concentration of about 5000 cells per well in 100µl of medium. The day after cells were treated with various concentrations of etoposide, ranging from 0,1µM up to 50µM, for 72 hours. In order to check the viability to each well 20µl of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (5µg/ml in PBS) was added and plates were incubated for about 4 hours in 37^0C. Then 100µl/well of lysis buffer (25ml ddH_2O, 25ml NN-dimethylformamid, 10g SDS, pH 4.7) was given and the plates were further incubated in 37^0C for overnight. Light absorption was read in ELISA reader at 570nm.

2.2.3. siRNA

Short double-strand RNA oligonucleotides (siRNA) (Ambion) for interference in the expression of Aurora A RNA were: 5'-UUCUUCCCAGCGCAUUCCUtt-3' (sense) and

5'-AGGAAUGCGCUGGGAAGAAtt-3' (antisense). The annealed oligonucleotides were dissolved in RNA-free water to obtain 100 μM stock solution. In vitro transfections were carried out in 6-well plates, using Oligofectamine reagent (Invitrogen) according to the manufacturer's instructions. Cells were harvested 24, 48, 72 and 96 hours upon transfection and the expression of Aurora A was controlled by Western blot analysis.

2.2.4 shRNA

The following hairpin oligonucleotides: AurA shRNA top: 5'-GATCCCCGTTCTTCCCAGCGCATTCCTTTCAAGAGAAGGAATGCGCTGGGAAG AATTTTTA-3' and AurA shRNA bottom: 5'-AGCTTAAAAATTCTTCCCAGCGCATTCCTTCTCTTGAAAGGAATGCGCTGGGAA GAACGGG-3', containing restriction sites for *BglII* and *HindIII*, were diluted in RNA-free water to final concentration 100 μM and annealed. Double-stranded hairpin oligonucleotides were digested and, after gel purification, cloned into *BglII-HindIII* restricted pSUPER vector (OligoEngine), which carries resistance to puromycin.

2.3. Results and comments

2.3.1. Aurora A is over-expressed in several cancer cell lines

Several cancer cell lines were tested for overexpression of Aurora A. Among them were the spleen cell lines TK6 and the mismatch-resistant variant MT1 (human hereditary spherocytosis), CACO (human colorectal adenocarcinoma) and SW837 (human rectum adenocarcinoma).

All cell lines showed elevated expression of Aurora A as evidenced by Western blot analysis. The amount of Aurora A was further increased upon synchronization of the cells in M phase with Nocodazole (Figure 1).

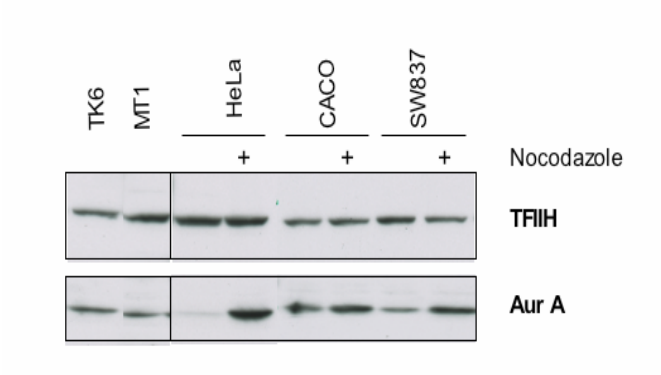


Figure 1. **Level of Aurora A protein in different cell lines.** Low and high level of Aurora A expression in exponentially growing and Nocodazole-treated HeLa cells, respectively, were used as control

2.3.2. Cells expressing higher amounts of Aurora A are more resistant to etoposide

In order to assess whether overexpression of Aurora A influences the sensitivity to etoposide we performed MTT assays on HeLa cells (as control) and on SW837 cells. The data show that SW837 cells, overexpressing Aurora A, display IC_{50} for etoposide that is about 10 times higher than HeLa cells (at 72h of etoposide treatment) (Figure 2).

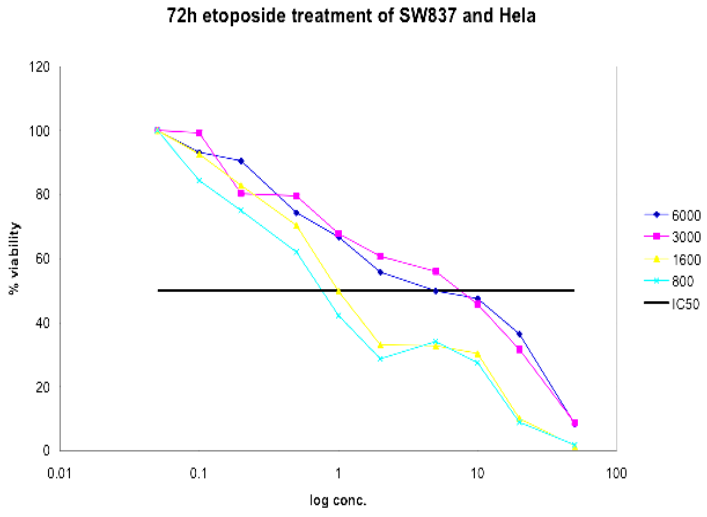


Figure 2. **SW837 are about 10 times more resistant to etoposide.** The cells were seeded in two different amount, namely 6000 and 3000 cells per well (SW837) and 1600 and 800 cells per well (HeLa).

Although the difference between HeLa and SW837 cells are certainly not limited to the overexpression of Aurora A, these data suggest that the latter might be an important factor in the observed 10-fold increased resistance of SW837 to etoposide.

2.3.3. siRNA approach to down-regulate AurA level in cancer cell lines

In order to test the quality of the small interference RNA to Aurora A that we designed we first tested its effect in HeLa cells, which are known to be easily transfectable with siRNA. Two concentrations of siRNA were used to check the selective down-regulation of Aurora A and eventual side effects (mortality of cells). Western blot analysis using specific antibodies revealed down-regulation of Aurora A expression to an extent of about 80% at 48h post-treatment, even with the lower concentration of oligonucleotide employed (50 nM), However, in order to obtain prolonged down-regulation of Aurora A expression to extent that is compatible with the duration of cell survival assays (MTT), higher amount of oligo was necessary (100 nM) (Figure 3).

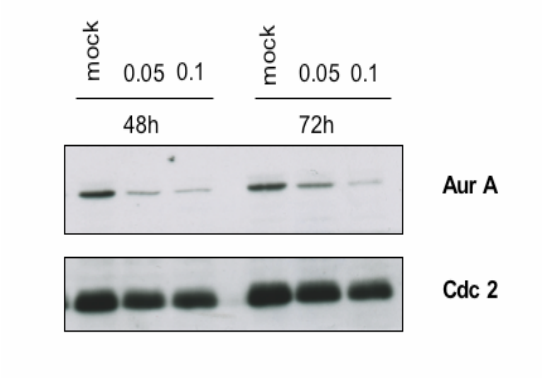


Figure 3. **Down-regulation of Aurora A by siRNA in HeLa cells.** The concentration of the Oligonucleotides is expressed in nanomolarity.

The same strategy was employed for SW837 cells. In this case, however, no down-regulation of Aurora A was observed due to the low transfection efficiency of these cells, as observed in control transfection experiments where an FITC labeled double-stranded oligonucleotide or the green fluorescent protein (GFP) vector were used. Several other methods of transfection were tested, such as Lipofectamine 2000 (Invitrogen), Interferin

(PolyPlus Transfections) and electroporation using siPORT buffer (Ambion) and XCell electroporator (BioRad) at various conditions of pulse length (75-200μs) and/or strength (200-400V). None of them, however, gave satisfactory result.

2.3.4. shRNA approach to down-regulate AurA level in cancer cell lines

Taking into account the obstacles mentioned in paragraph 2.3.3 we decided to down-regulate Aurora A expression using an shRNA approach followed by cloning of shRNA-carrying cells and antibiotic selection. This approach seems to posses several advantages: (1) even low transfection efficiency would be enough to select single, transfected with shRNA, cells; (2) the level of Aurora A down-regulation in cells derived from a single clone will be identical; (3) different clones, may display different down-regulation level, which would enable us to closer examine the effect of etoposide and sensitivity to it in regard to amount of Aurora A protein present.

References:

Bischoff J.R., Anderson L., Zhu Y., Mossie K., Ng L., Souza B., Schryver B., Flanagan P., Clairvoyant F., Ginther C., Chan C.S.M., Novotny M., Slamon D.J., Plowman G.D., "A homologue of *Drosophila* aurora kinase is oncogenic and amplified in human colorectal cancers", 1998, *The EMBO Journal*, 17: 3052-3965

Hata T., Furokawa T., Sunamura M., Egawa S., Motoi F., Ohmura N., Marumoto T., Saya H., Horii A., "RNA interference targeting Aurora Kinase A suppresses tumor growth and enhances the taxane chemosensitivity in human pancreatic cancer cells", 2005, 65: 2899-2905

Kamada K., Yamada Y., Hirao T., Fujimoto H., Takahama Y., Ueno M., Takayama T., naito A., Hirao S., Nakajima Y., "Amplification/overexpression of Aurora A in human gastric carcinoma: potential role in differentiated type gastric carcinogenesis", 2004, *Oncol. Rep.,* 12: 593-599

Royce M.E., Xia W., Sahin A.A., Katayama H., Johnston D.A., Hortobagyi G., Sen S., Hung M.C., "STK15/Aurora A expression in primary breast tumors is correlated with nuclear grade but nit with prognosis", 2004, *Cancer,* 100: 12-19

Ulisse S., Delcros J-G., Baldini E., Toller M., Curcio F., Giacomelli L., Prigent C., Ambesi-Impiombato F.S., D'Armiento M., Arlot-Bonnemains Y., "Expression of Aurora kinases in human thyroid carcinoma cell lines and tissues", 2006, *Int. Journal of Cancer,* published on-line 13.02.2006

Zhou H., Kuang J., Zhong L., Kuo W.L., Gray J.W., Sahin A., Brinkley B.R., Sen S., "Tumour amplified kinase STK15/BTAK induces centrosome amplification, aneuploidy and transformation", 1998, *Nature Genetics,* 20: 189-193